Isolation of Novel Antibiotic Producing Microorganisms from Invertebrates

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List of Abbreviations

AIA Actinomycete Isolation Agar

AIB Actinomycete Isolation Broth

AMR antimicrobial resistance

Br Broad

$CaCO_3$ Calcium carbonate

CD_3OD Methanol-D_4

CLS Cell Lysis Solution

COSY Correlation Spectroscopy

D doublet

1D-NMR 1-dimensional methods

2D-NMR 2-dimensional methods

DIZ diameter of inhibition zone

DMSO-D_6 deuterated Dimethyl sulfoxide-D_6

DNA Deoxyribonucleic Acid

D of D doublet of doublet

ESBL Extended Spectrum Beta-Lactamases

$FeSO_4.7H_2O$ Ferrous sulfate heptahydrate

FT-IR Fourier Transform Infrared

GNB Glucose Nitrate Broth

HCAI Health Care Associated Infections

HMBC Heteronuclear Multiple-bond Correlation Spectroscopy

HMQC Heteronuclear Multiple-Quantum Correlation

HPLC High-Performance Liquid Chromatography

IC Inhibitory concentration

ITS Internal transcribed spacer

KCl Potassium chloride

K_2HPO_4 Dipotassium phosphate

LC Liquid Chromatography

MEA Malt Extract Agar

MeOH Methanol

Mg Milligram

MIC Minimum Inhibitory Concentration

Ml Millilitre

Mg $SO_4.7H_2O$ Magnesium sulfate heptahydrate

MHA Muller Hinton Agar

MRSA Methicillin-Resistant Staphylococcus Aureus

MS Mass Spectrometry

MW Molecular weight

m/z mass-to-charge

$NaNO_3$ Sodium nitrate

NaOH Sodium hydroxide

NB Nutrient Broth

NH_4Cl Ammonium chloride

$(NH_4)_2SO_4$ Ammonium sulfate

NMR Nuclear Magnetic Resonance

NP-HPLC Normal Phase High-Performance Liquid Chromatography

NRPS Non-ribosomal peptide synthetases

PCR polymerase chain reaction

PCR-RFLP polymerase chain reaction-restriction fragment length polymorphism

PDA Potato Dextrose Agar

PKS Polyketide synthetases

Ppm Parts Per Million

Rep-PCR Repetitive intergenic DNA sequences

RP-HPLC Reverse Phase High-Performance Liquid Chromatography

RNA Ribonucleic Acid

SEC Size exclusion chromatography

S singlet (1H NMR)

S Sharp (IR)

Sms Small medium sharp

SNB Starch Nitrate Broth

SNB2 Starch Nitrate Broth and Nutrient Broth 2:1

SNHB Starch Ammonium Broth

SNHB2 Starch Ammonium Broth and Nutrient Broth 2:1

Ss Small sharp

TFA Trifluoroacetic Acid

TLC Thin-Layer Chromatography

TMS Tetramethylsilane

TSA Tryptone Soya Agar

TSB Tryptone Soya Broth

UV Ultra Violet

YEB Yeast Extract Broth

YMA Yeast Malt Extract Broth

µg Microgram

µl Microlitre

Abstract

Because of the increased incidence of antibiotic resistance there is an increasing need to isolate new microorganisms producing new antibiotics. Because conventional sources such as soil have been exhausted, attention has been turned to unexplored sources. The aims of this research were to investigate terrestrial and aquatic invertebrates as a source of antibiotic producing microorganisms, to isolate the organisms and screen them for activity against Gram-positive and Gram-negative bacteria. Those with activity would be further characterised. Forty-seven antibiotic producers were isolated from 35 samples of different terrestrial and aquatic invertebrates using media designed to isolate streptomycetes. Identification by PCR and sequencing of 16S and 18S rRNA showed that the majority were in fact fungi, mainly *Penicillium* spp. and only 5 were *Streptomyces* spp. Three strains isolated from *Helix aspersa* (ES1), *Lumbriculus variegata* (La1a) and *Baetis* larvae (M1 11) that had strong activity against Gram-positive and Gram-negative bacteria including Methicillin-resistant *Staphylococcus aureus* (MRSA) were chosen for further study. PCR and sequencing of rRNA and Internal Transcribed Spacer (ITS) regions confirmed that the strains were closely related and were probably strains of *P. chrysogenum*. The organisms were grown in liquid media and on agar and the active components were extracted with ethyl acetate and fractionated by HPLC. As facilities for large scale liquid cultures were not available large scale preparation was performed from agar medium and the active fractions. HPLC showed that the extracts were complex and contained more than one active component. The purified fractions were chemically characterised by NMR, FTIR and MS. The results showed that the active components were novel flavones and isoflavones. Such compounds have extracted from plants and only previously been shown to be produced by endophytic fungi. The minimal inhibitory concentrations (MIC) against test bacteria and inhibitory concentration IC50 (IC50 is the concentration of a compound at which 50 % of human cancer cell tissue cultures are killed) showed potential for use in chemotherapy as all purified fractions except Es1 F3/3 and Es1 F6 were found to have IC_{50} values>100 µg/ml, High IC_{50} and low MIC (0.39 to 50 µg/ml) may indicate fractions suitable for testing for treatment of human infections. The results showed that the invertebrates were indeed a source of antibiotic producing organisms.

Whether the organisms were acquired by the invertebrates from the environment or whether they were in a symbiotic association needs further study.

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Chapter 1

1. INTRODUCTION

1.1 Definition of antibiotics and antimicrobial agents

By definition, an antibiotic (from the Greek: Anti; "against", and – bios, "life") is a substance or compound formed by one organism which at low concentrations can inhibit the growth of or kill other organisms and is usually used to describe substances that act on microorganisms (Agbor *et al*., 2011, Sharma *et al*., 2013)**.** Antimicrobial chemotherapeutic agents also includes agents that can be can be chemically synthesized. Many antibiotics are now made chemically or are chemically modified (semi-synthetic) so that the difference between the two terms is often blurred. Antibiotics are therefore described as naturally occurring, semi-synthetic and synthetic compounds with antimicrobial activity that can be administered parentally, orally or topically (Lee *et al., 2*001).

Microbes produce a very large number of different organic compounds, often with low molecular weights, which makes up a very diverse chemical and biological ecology. It has been suggested that, at low concentrations, most of these compounds play a crucial role in the alteration of metabolic function within natural microbial environments (Yim *et al*., 2006). In these environments, organisms that produce antimicrobial compounds have higher survival rates because the compounds produced can destroy or inhibit the growth of other existing competing bacteria (Kumar *et al*., 2012b).

Before the discovery of antibiotics there were few options for treating infections caused by microorganisms. The first chemotherapeutic agent was arsphenamine (Salvarsan), an organoarsenic compound that was introduced by Paul Ehrlich in 1910 as a treatment for syphilis although it was extremely toxic (Williams, 2009). The sulphonamide drugs which were introduced in the late 1930's were effective antibacterial agents and were important in the UK during World War II, However, they had serious side effects and they were rapidly superseded by the introduction of penicillins after their discovery by Fleming in 1928 and their purification by Florey and his co-workers in the early 1940's (Davenport, 2011). The discovery of

antibiotics in the 20th century was considered a great achievement, however, now there is a concern in the increase of antibiotic resistance within hospitals, communities and the environment related to their use (Davies and Davies, 2010). Indeed there are some infections that are resistant to most if not all antibiotics (Livermore, 2009, Fifer *et al*., 2016).

The discovery of penicillin started the "golden age" of antimicrobial therapy. Newer antibiotics both from natural origins and synthetic were discovered mainly in the period between 1940 to 1990. During this period most of the antibiotics including *β*-lactams, tetracyclines, chloramphenicol, aminoglycosides, macrolides, glycopeptides, streptogramins and quinolones with different mechanisms of action on and affecting different targets in bacteria were introduced into clinical practice. Since the "golden age" most "new" antibiotics/antibacterial agents have been produced by chemically modifying pre-existing antibiotics in order to make various generations with enhanced efficacy and spectra of activity (Bbosa *et al*., 2014). Since the discovery and development of penicillin in the 1940s, further research has been carried out, in particular the *Streptomyces* spp., to develop antimicrobials that can act against pathogenic bacteria. Currently, there are more than 350 agents that have been introduced to the world industry as antimicrobials (Silber *et al*., 2016). The research and study of antibiotics/antibacterial agents have greatly transformed the treatment of bacteria related infections that used to overwhelm millions prior to the "golden age". (Bbosa *et al*., 2014). However there have been few new antibiotics and few new targets identified over the last 30 -40 years.

1.2 The Basic characteristics of antibiotics

An ideal antibiotic should fulfill certain criteria. Concerning toxic side effects it must be relatively toxic to microbes but nontoxic to host cells. It is preferred to be microbiocidal rather than micro-biostatic. Moreover, it is recommended to be reasonably soluble and functional even when highly diluted in body fluids. Some of the most valuable characteristics are that resistance to the antibiotic does not develop quickly, that it does not cause allergic reactions in host tissues or be prematurely excreted or broken-down. Finally it is preferable to be of reasonable price (Talaro, 2007).

The antibiotic concept stated above indicates that small molecules are produced naturally by bacteria and/or fungi in adequate quantities to suppress or destroy other microorganisms thus reducing competition in natural environments. This was demonstrated in the laboratory when strains acquired from starved environments were exposed to rich, unnatural growth conditions, this triggered the production of compounds in large quantities (Yim *et al*., 2006).

The production of appropriate amounts of compound for preliminary chemical and clinical studies requires efforts in microbiology (e.g. use different media, different incubation period, etc.), chemistry (e.g. use of different solvents for extraction, use of different chromatography technique to purify the active compounds etc.), and the isolation of strains of the microorganism that produce higher yields. Therefore, small molecules produced in low concentrations are not easily detected by these methods.

One of the more interesting discoveries from genome sequencing efforts is the demonstration that, based on current knowledge of biosynthetic pathways and their organization, some organisms possess the genetic capability to produce variable numbers of different small bioactive molecules (Bentley *et al.*, 2002, Ikeda *et al.*, 2003). The genome sequence of *S. coelicolor* revealed 18 clusters of genes for secondary metabolite synthesis include some that had not previously been identified. These include polyketide synthases type I and II, non-ribosomal peptide synthetases (NRPSs), chalcone synthases and others (Bentley *et al.*, 2002).

1.3 Mechanisms of antibiotic action

The classification of antibiotics is based on either the cellular component or the system they affect, in addition to whether they trigger cell death (bactericidal drugs) or simply inhibit cell growth (bacteriostatic drugs). Currently bactericidal antimicrobials, inhibit DNA synthesis, RNA synthesis, cell wall synthesis, membrane permeability, intermediary metabolism or protein synthesis (Figure 1.1; Kohanski *et al*., 2010).

On the basis of their mechanism of action, antibiotics are classified as:

Those affecting the biosynthesis of the bacterial cell wall, leading to loss of viability and often cell lysis (e.g. penicillins, cephalosporins, bacitracin, and vancomycin) but often only on actively growing cells.

Those that act directly on the cell membrane, disrupting its barrier function and causing leakage of intracellular components (e.g. polymyxins).

Those that affect protein biosynthesis (e.g. chloramphenicol, tetracyclines, erythromycin, streptomycin, gentamycin)

Those that interfere with nucleic acid biosynthesis (e.g. rifampicin, novobiocin, quinolones).

Those that block specific steps in intermediary metabolism (e.g. sulfonamides, trimethoprim; Husain *et al*., 2010).

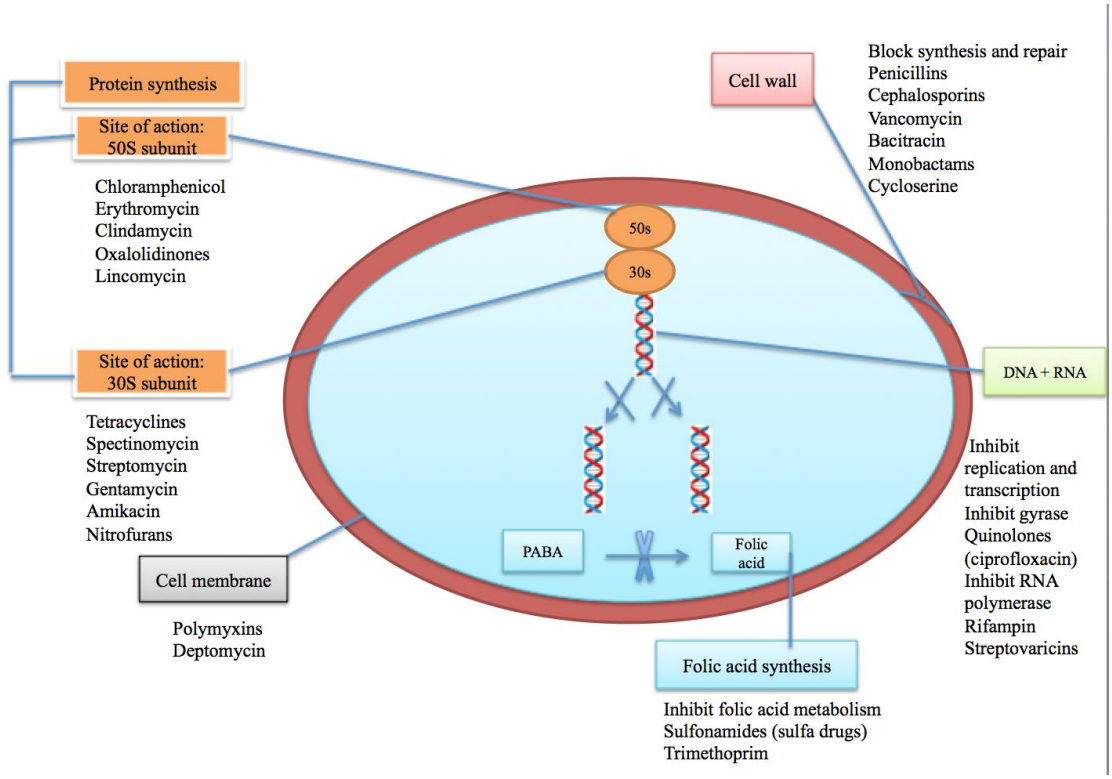


Figure 1.1: Mode of action of antibiotics on cell components
Modified from Bbosa *et al*. (2014)

1.4 Microbial resistance to antibiotics

The ability of a microorganism to withstand antibiotic effects is referred to as antibiotic resistance. It evolves through natural processes such as natural selection *via* random mutation, or it could also be engineered by applying an evolutionary stress on a population. The antibiotic action is an environmental pressure such that resistant bacteria will survive and continue to reproduce. The specific trait is then transferred to the offspring, leading to a fully resistant generation. As antibiotic resistance protects antibiotic-producing organisms from their own products and other originally susceptible organisms from competitive attack, it is has coevolved with antibiotics (Sarmah *et al*., 2006, Kemper, 2008).

The resulting genes can then be transferred by horizontal gene transfer between individuals *via* transduction, conjugation or transformation (Morell, 1997, Konaté *et al.,* 2013). Antibiotic resistance can be introduced into a microorganism by artificially implanting genes into it (Gaude and Hattiholli, 2013).

In both humans and veterinary medicine, antibiotics are used to cure and prevent disease. The development of resistant bacterial strains impacts on the use of antibiotics as treatment in both humans and animals and represents a major health concern. The extensive use of veterinary pharmaceuticals is supposed to be an important public health risk that results from the emergence and spread of resistant bacteria. Particularly, the application of veterinary antibiotics to food animals is likely to increase the selection for strains resistant to antibiotics used in human medicine (Marshall and Levy, 2011). These strains may be transmitted to humans through direct contact with animals or via the food chain to the consumers, in food animal adding antibiotics encompasses a wide variety of nontherapeutic purposes that include growth promotion. For example, growth promotion in animal husbandry using the streptothricin antibiotic nourseothricin led to plasmid-borne resistance to streptothricin in *Escherichia coli* from nourseothricin fed pigs, from workers in pig farms and from their family members. Furthermore, resistant *E. coli* were found in people without any contact to pig farms (as part of the gut mocrobiota and even causing urinary tract infections). However, these individuals lived in villages and towns of the region where nourseothricin was used on pigs (Hummel *et al*., 1986).

The risks that originate from excessive use of antibiotics lead to environmental contamination with antibiotic resistant bacteria and antibiotics. Furthermore, the impacts on the biotic environment are a major concern (Kemper, 2008).

1.4.1 Mechanisms of resistance

There are five main mechanisms of antibiotic resistance (Figure 1.2):
Production of inactivating enzymes
Changes in the target site
Induction of alternate pathways (bypass pathways)
Failure to activate the antibiotic to its active state
Exclusion of the antibiotic from the target site or active efflux
The latter may happen by restriction of access through porins (only in Gram-negatives) or by active secretion (Scott, 2009).
The major mechanisms of antibiotic resistance are classified as **modifications of the Antibiotic Molecule** (chemical alterations of the antibiotic and destruction of the antibiotic molecule)**, Target site change (**target protection and modification of the target site (mutations of the target site, enzymatic alteration of the target site and complete replacement or bypass of the target site)) and **exclusion and active efflux of the antibiotic (**decreased permeability and efflux pumps (Munita and Arias, 2016)

Modifications of the Antibiotic Molecule
Production of enzymes by resistant bacteria to inactivate the antimicrobial compounds is one of most successful strategies to survive in case of exposure to antibiotics by adding chemical moieties to the antimicrobial molecule or that destroy molecule itself.

1. Chemical alterations of the antibiotic
Gram-positive and Gram-negative bacteria are well known mechanism of acquired antibiotic resistance is synthesize enzymes that introducing chemical changes to the antimicrobial molecule. Aminoglycosides are inactivated by modifying enzymes that phosphorylate (aminoglycoside phosphoryltransferase, acetylate (aminoglycoside acetyltransferase, or adenylate (aminoglycoside adenytransferase; Poole, 2005a).
One of the best example of the resistance due to chemical alteration of the antibiotic is

modifying enzymes that inactivate aminoglycosides by covalently modifying the hydroxyl or amino groups of the aminoglycoside molecule. These enzymes are usually carried on mobile genetic elements, but chromosomal genes coding for modifying enzymes have also been found in in some bacterial species, such as aminoglycoside acetyltransferases in *Providencia stuartii, Enterococcus faecium and Serratia marcescens* (Ramirez and Tolmasky, 2010)

2. Destruction of the antibiotic molecule

Degraded β-lactam ring by the action of β- lactamases is the main mechanism of β-lactam resistance. In general, Gram-positive bacteria produce a large amount of β-lactamase, which is secreted extracellularly, in Gram-negative bacteria, β-lactamases are found in relatively small amounts and are located in the periplasmic space, between the inner and outer cell membranes. In this group, beta-lactamases destroy the amide bond of the β-lactam ring resulting in inactivation of the antibiotic (Cesur and Demiroz, 2013, Soares *et al*., 2012).

Target site change

Bacteria avoid the action of the antimicrobial molecule by interfering with their target site, this strategy common in bacteria to develop antibiotic resistance; these include protection of the target and modifications of the target site

1. Target protection

In this mechanism the target site is protected by prevention of the antibiotic reaching its binding site. Clinically relevant genes coding for proteins that mediate target protection are usually carried by mobile genetic elements but some have been found on the bacterial chromosome. Tetracycline resistance determinants Tet(M) and Tet(O) are classic examples of the target protection mechanism. Tet(M) and Tet(O) are now widely distributed among different bacterial species, Tet(M) at first was described in *Streptococcus* spp. and Tet(O) in *Campylobacter jejuni*, possible because both have been found in several plasmids and in broad-range conjugative transposons (Connell *et al*., 2003). TetO and TetM interact with the ribosome and dislodge the tetracycline from its binding site.

2. Modification of the target site

There are different ways to modify of the target site**,** mutations in the genes encoding the target site, enzymatic alterations of the binding site (e.g. addition of methyl groups), and/or replacement or bypass of the original target. All this causes decrease in the affinity of the antibiotic for the target site (Munita and Arias, 2016).

2.1 Mutations of the target site

Crucial target site changes include topoisomerases II and IV (attacked by quinolones, (Fàbrega *et al*., 2008), β subunit of DNA-dependent RNA polymerase (usually a target of rifamycins, (Depardieu *et al*., 2007). The development of rifampin resistance is an example of mutations of the target site. The rifampin binding pocket is located in the β subunit of the RNA polymerase (encoded by *rpoB*), the antibiotic molecule interrupts transcription after binding to its target site by directly blocking the path of the nascent RNA (Campbell *et al*., 2001). Single-step point mutations resulting in amino acid substitutions in the *rpoB* gene causing high-level rifampin resistance have been reported, these mutations decrease the affinity of the antibiotic for its target (Floss and Yu, 2005).

2.2 Enzymatic alteration of the target site

In one of the macrolide resistance mechanisms, bacteria develop resistance through the modification and methylation of the ribosomal RNA binding site of the antibiotic by an enzyme encoded by the *e*rythromycin *r*ibosomal *m*ethylation genes (*erm* genes). These enzymes are capable of mono- or dimethylating an adenine residue in position A2058 of the domain V of the 23rRNA of the 50S ribosomal subunit, which this change will cause low affinity of the antimicrobial molecule to its target site (Weisblum, 1995, Leclercq, 2002).

2.3 Complete replacement or bypass of the target site

This is observed in β-lactam resistance in MRSA by the acquisition of a foreign gene (likely from *Staphylococcus sciuri*) designated *mecA* often located in a large DNA fragment designated staphylococcal chromosomal cassette mec (*SCCmec*), which encodes a novel penicillin-binding protein (PBP2a), which has low affinity for β-lactam antibiotics and enables the bacteria to assemble the cell wall in the presence of the β-lactam antibiotics (Ba *et al*., 2013, Munita and Arias, 2016, Hawkey, 1998).

Another of resistance mechanism whereby the normally inhibited metabolic pathway is bypassed by overproducing the antibiotic target by increasing the amount of target site available and the antibiotic not overwhelming the bacteria. Trimethoprim-sulfamethoxazole resistant bacteria are a good example of this mechanism. This antibiotics interact with the folic acid synthesis, two enzymes involves in folic acid synthesis, dihydropteroic acid synthase (DHPS) inhibited by sulfamethoxazole and dihydrofolate reductase (DHFR) inhibited by trimethoprim, due to mutations in the promoter region of the DNA encoding these enzymes result in the production of increased quantities of the DHPS and DHFR, this allows bacteria to survive and trimethoprim-sulfamethoxazole do not inhibit folic acid production (Flensburg and Skold 1987, Eliopoulos and Huovinen 2001)

Exclusion and active efflux of the antibiotic

Porins are protein structures located in the outer bilipid membrane of Gram-negative organisms. They control what passes into and out of the cell based on molecular size and charge. Mutations in the genetic elements encoding porins (permeability mutations) may prevent entry of one or multiple antibiotics (Poole, 2005b). *Escherichia coli* K-12 produce large amount of OmpF and OmpC, two major outer membrane proteins regulated by the *ompB* locus. Mutants lacking the OmpF proteins have increased antibiotic resistance (Jaffe *et al*., 1982). In *P. aeruginosa* loss of OprD proteins due to mutation in the *oprD* gene causes a significant decrease in the sensitivity of *P. aeruginosa* to carbapenems (Sanbongi *et al*., 2009, Azimi *et al*., 2015).

Alternatively, organisms may actively excrete antibiotics. McMurry *et al*. (1980) were the first to describe efflux pumps as a mechanism of resistance to tetracycline. Tet efflux pumps are able to actively expel tetracycline from *E. coli* by plasmid-encoded proteins. Later, many efflux pumps have been described (in both gram-negative and gram-positive pathogenic bacteria) and these pumps may be specific for certain antibiotic (substrate-specific) such as *tet* determinants for tetracycline and *mef* genes for macrolides in *pneumococci* or for multi antibiotics (broad substrate specificity), its usually found in multi drug resistance bacteria (Poole, 2005b).

Efflux pump encoding genes can be located in mobile genetic elements such as Tet efflux pumps encoded by the *tet* gene (mention above) and MsrA is a plasmid-borne determinant that was originally described in *S. epidermidis* or in the chromosome

such as MsrC efflux pump, chromosomal-encoded described in *E. faecalis* that confers resistance to erythromycin and other macrolide and streptogramin B antibiotics and Lsa efflux pumps encoded by the chromosomal gene *lsa* that causes *E. faecalis* resistance to lincosamides and streptogramin A (Singh *et al*., 2002, Poole, 2005b). The Mef pumps are mainly found in *S. pyogenes* and *S. pneumonia* encoded by the mef genes (mefA and mefE), mefA located in the chromosome and usually carried o a n a transposon (Tn1207), MefE has been fond located in different regions of the bacterial chromosome in fragment of DNA called *m*acrolide *e*fflux *g*enetic *a*ssembly element, these pumps cause resistance to macrolide antibiotics Ross *et al*., 1990.

Efflux pumps have been divided into five major families on the basis of structural conformation, sequence similarity, energy source, range of substrates they are able to extrude (single or multiple) and in the type of bacterial organisms: the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily, the resistance-nodulation-division (RND) family, the small multidrug resistance (SMR) family, the major facilitator superfamily (MFS) and the multidrug and toxic compound extrusion (MATE) family (Blanco *et al*., 2016, Munita and Arias, 2016).

The MFS, ABC, SMR, and MATE families are broadly spread in Gram-positive and Gram-negative bacteria, whereas the RND superfamily is specific to Gram-negative bacteria Blanco *et al*., 2016. Efflux pump group belong to MFS family is most relevant to Gram-positive bacteria, PmrA from *S. pneumoniae* and NorA from *S. aureus* the best described of MFS family (Lorca *et al*., 2007, Ubukata *et al*., 1998), also Tet efflux pumps belonging to the MFS family Poole, 2005b. Msr(A) efflux pumps resulting in macrolide resistance in gram-positives, belonging to the ABC superfamily Reyes *et al*., 2007. Examples of the RND family are AcrAB-TolC in *Enterobacteriaceae* and MexAB-OprM in *P. aeruginosa* (multidrug resistance efflux pumps) Visalli *et al.*, 2003, Dean *et al*., 2003.

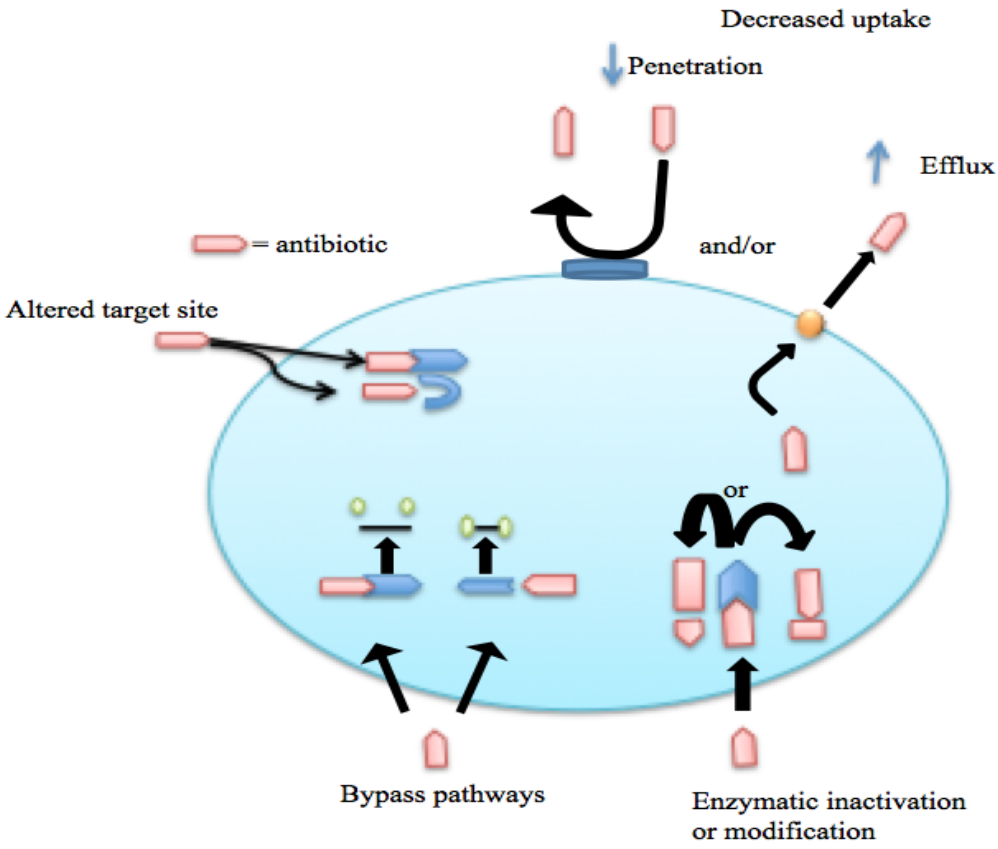


Figure 1.2: Four major biochemical mechanisms of antibiotic resistance
Modified from Hawkey (1998)

1.4.2 Acquisition of resistance

Direct mutation of chromosomal genes causes resistance, and non-fatal mutations are passed to all offspring (which acquire a selective advantage in the presence of the antibiotic). Alternatively, bacteria may acquire external genetic material from other bacteria (even bacteria of different species) by three different mechanisms. Conjugation like the direct transfer by type IV, F- or P- ("sex") pili. In the process of Conjugation is a process in which a mobile or conjugative genetic element (such as a plasmid or transposon) is transferreded from the contributor cell. In the appropriate environment, plasmids can reproduce each time an organism divides and can be lost as easily as they are acquired. Transposons are large genetic elements usually containing various genes essential to confer phenotypic resistance (Holmes, 1996). Transformation is one of the processes to transfer genetic elements material including antibiotic resistance gens into a bacterial cell, free DNA from dead bacteria and even mammalian cells can be taken up and merged into bacterial chromosomes by the

process of transformation (Scott, 2009). The third mechanism for horizontal genetic transfer is transduction involving transfer of DNA from one bacterium into another via bacteriophages. Table 1.1 shows some examples of acquired resistance (Abebe *et al*., 2016).

Table 1.1: Examples of acquired antibiotic resistance

Modified from Abebe *et al*. (2016), Lyon and Skurray (1987)

Acquired resistance by	**Resistance observed**	**Mechanism involved**
Mutations	*Mycobacterium tuberculosis* resistance to rifamycins	Point mutations in the rifampin-binding region of *rpoB*
	Resistance of many clinical isolates to fluoroquinolones	Predominantly mutation of the quinolone-resistance-determining-region (QRDR) of GyrA and ParC/GrlA
	E.coli, Hemophilius influenzae resistance to trimethoprim	Mutations in the chromosomal gene specifying dihydrofolate reductase
Horizontal gene transfer	*S. aureus* resistance to methicillin (MRSA)	Via acquisition of *mecA* genes which is on a mobile genetic element called "staphylococcal cassette chromosome" (SCCmec) which codes for penicillin binding proteins (PBP2a)
	Resistance of many pathogenic bacteria against sulfonamides	Mediated by the horizontal transfer of foreign genes or parts of it
	E. faecium and *E. faecalis* resistance to vancomycin	Via acquisition of one of two related gene clusters VanA and Van B, which code for enzymes that modify peptidoglycan precursor, reducing affinity to vancomycin.
	S. aureus resistance to gentamycin	Mediated by the conjugational transfer of self- transmissible (Tra+) plasmids transferred between apparently nonlysogenic strains of *S.aureus*

1.5 Importance of resistance in Healthcare acquired infections

Patients undergoing treatment within the healthcare are exposed to infections that are recognized worldwide to contribute to morbidity and mortality. Some patients such as children, elderly and patients with weakened immune systems are more fragile and are at greater risk of acquiring these infections. The close cohorting of patients with reduced resistance to transmissible diseases provides an ideal environment for antibiotic resistant bacteria to spread from one patient to another. Health Care Associated Infections (HCAI) are infections resulting from medical care, these infections can be acquired by various means such as through long stays in hospitals; not using sterile equipment; failure of healthcare personnel to wash hands, overuse of antibiotics and failure to maintain aseptic techniques (Tabatabaei *et al*., 2015). HCAI effecting people with direct result of treatment in, or contact with health care setting, also people acquired infection outside a healthcare and brought into a healthcare by patient, staff, or visitors and transmitted to others. HCAI Pose a significant risk to patients, staff and visitors to health and social care premises. According to reports from Health Protection Agency, 2011; Public Health England (PHE), 2012 approximately 1 in 8 patients are affected by a HCAI which can incur costing to the NHS in excess of £1 billion per year and estimated that 5000 people die a year from such infections. HCAI covers a wide range of infections, the most well known include those caused by MRSA, Methicillin-sensitive *S. aureus* (MSSA), *Clostridium difficile (C. diff)* and *E. coli* (Carr, 2016). As a result, Public Health England (PHE) advise and support the NHS in efforts to prevent and control HCAI.

PHE annual epidemiological commentary dated 2016/17 on HCAIs reports that across England the rate of MRSA bacteraemia remained steady at 1.5 cases per 100,000 population in 2016/17 and over the last three financial years (2014/15 to 2016/17) dropped by 7.1% in 2014/15 compared to the previous financial year. The rates of MSSA and *E.coli* bacteraemia both increased between 2015/16 and 2016/17, at 19.3 cases per 100,000 population in 2015/16 to 20.9 in 2016/17 in MSSA, at 69.7 cases per 100,000 population in 2015/16 to 73.9 cases per 100,000 population in 2016/17 in *E. coli*. The rate of *C. difficile* infection has reduced from 25.8 cases per 100,000 population in 2015/16 to 23.4 cases per 100,000 population in 2016/17. MRSA bacteraemia infection source from intravenous catheter or line have seen considerable

decreased in the percentage between 2007/08 and 2016/17,this is likely due to interventions aimed at improving care in the acute setting. Nevertheless, infection of MRSA bacteraemia from catheters and lines source have seen increase between 2015/16 and 2016/17 and may require further effort to reduce it again.
This is not unique problem to the UK, it is world wild problem for example according to report from Centers for Disease Control and Prevention (CDC) in United States, more than 2 million infections with 23 000 deaths annually causes by AMR (Holmstrup and Klausen, 2018) and in Europe, AMR causes approximately 25 000 deaths per year and cost over €1.5 billion in healthcare expenses related to these infection (Ronnerstrand and Lapuente, 2017). In India the situation is even more alarming with estimated that more than 58 000 infants died in the year 2013 as a result of AMR infections (Rather *et al*., 2017). In Thailand, more than 38 000 death per year (Jepsen and Jepsen, 2016).
Plans to overcome antibiotic resistance within the healthcare professions include multidisciplinary collaboration in operating local policies on use of antibiotics and infection control measures, early detection and reporting of the antibiotic resistant strains, enhanced surveillance, and make good strategies to control transmission of epidemic resistant bacteria (Struelens, 1998)

1.6 The need for antibiotic research and discovery

One solution to the emergence of bacterial resistance is the development of new antibacterial agents for substitution for ineffective drugs, therefore the search for new antibiotics either of unique structure or improved activity from this apparently endless source (microorganisms) continues to be of extreme importance in research programs around the world, especially with the increased prevalence of resistant pathogens. In the 21st century, antimicrobial resistance (AMR) described as one of the major threats to individual and population, AMR have repeatedly underlined the urgent need for action by national and international organizations (Davies and Gibbens, 2013, O'Neill, 2014)
Infectious diseases are the second highest causes of death worldwide despite the progress made in the process and production of antibiotics (Silber *et al*., 2016).
The appearance of infectious caused by multi-drug resistant strains and important clinical indication of drug resistance has led to increased interest in the research of

antimicrobial resistance from various aspects. To solve this problem, further research is required to discover new more efficient antimicrobial agents. Fungi, particularly from the genera *Aspergillus* and *Penicillium* are eukaryotes that serve as a reservoir of bioactive metabolites which can be used for the purpose of medicinal study (Compaore *et al*., 2016a)

In the coming years, advancing screening programs for the selection of different natural and modified chemical compounds will be a necessity to discover new antibiotics active against resistant bacteria (Sanchez *et al*., 2010**).** There is also an increasing demand for antibiotics. For example, in a study carried out by "marketresearch.com", China's demand for penicillin had grown exponentially between 1990 and 2000 and this demand was forecast to continue to grow and expand over the subsequent years (Dayalan *et al*., 2011).

1.7 Natural products

Nature has been a source of therapeutic agents for thousands of years, and an impressive number of modern drugs have been derived from natural sources, many based on their use in traditional medicine (Murugan and Mohan, 2011). Products came from natural origins can be called "natural products".

Natural products come from four major sources

(i) An entire organism (e.g., a plant, an animal, or a microorganism) that has not been subjected to any kind of processing or treatment other than a simple process of preservation,

(ii) Part of an organism (e.g., leaves or flowers of a plant, or an isolated animal organ),

(iii) An extract or an exudate of an organism, and

(iv) Isolated pure compounds from plants, animals, or microorganisms, (e.g., alkaloids, flavonoids, glycosides, steroids, sugars, terpenoids, etc.)

Nevertheless, in most cases the term natural products refers to secondary metabolites (small molecules produced by an organism that are not strictly necessary for the living of the organism). Natural products can be from any terrestrial or marine source: plants, animals, or microorganisms. The databases of natural products (Super Natural II database) comprises 325 508 natural compounds from almost all part of the world

(Xie *et al*., 2015) The natural product drug discovery process in general includes the testing of extracts of plant, marine or microbial origin in adequate *in vitro* assays target based, followed by bioassay- guided fractionation of the active extracts and isolation and purification of active constituents. Those constituents showing significant *in-vivo* activity in appropriate animal models are considered as lead molecules, which may be chosen as candidates for preclinical development.

1.8 Secondary metabolites

Due the fact that they are not produced during normal growth antibiotics are categorized as secondary metabolites. Characteristically they are not usually produced during the phase of rapid growth (trophophase), but are synthesized during a subsequent phase of secondary metabolism that starts when growth is limited by the exhaustion of one key nutrient source. Secondary metabolites are substances that are indirectly involved in normal growth, development, or reproduction of an organism; these compounds are not involved in a physiological role during exponential phase of growth.

Secondary metabolites are organic compounds produced by strain of certain microbial species and by some plants, they are regarded as a crucial component in the discovery and development of drugs (Shaala and Youssef, 2015)**,** these compounds are varied and sophisticated chemical structures, there are many metabolites with an enormous range of biological activities but the antibiotics are the best known secondary metabolites. Most secondary metabolites of commercial importance are produced by actinomycetes, especially genus *Streptomyces,* and by fungi (Gonzalez *et al*., 2003). Endophytic fungi which can be found in the tissue of living plants seem to contribute secondary metabolites as part of the plants normal functions (Devi *et al*., 2012). Spore pigments also have structures similar to those of other secondary metabolites and are synthesized by the same kinds of mechanisms (Nakano *et al*., 2000). The genes coding for the enzymes responsible for the synthesis of secondary metabolites are often clustered. These clusters include the genes for the actual biosynthesis, as well as determinants for regulation and self-resistance (Pissowotzki *et al.*, 1991). Sometimes the genes are located on plasmids. It is apparent that horizontal transfer of genes coding for secondary metabolites can take place in soil (Egan *et al.*, 1998, Ōmura *et al.*, 2001). The gene clusters are frequently polyketide synthetases (PKS) or non-ribosomal peptide synthetases (NRPS), Wang *et al*., 2014a have been reported the

widespread occurrence of NRPS and PKS genetic machinery across the three domains of life (bacteria, archaea, and eukarya) with the discovery of 3,339 gene clusters from 991 organisms, by examining a total of 2,699 genomes.

The production of secondary metabolites is affected by the availability of nutrients. In fermentation experiments, the production of antibiotics is increased by the presence of a non-preferred carbon source, or by phosphate starvation (McDowall *et al.*, 1999). By optimizing both the nutrition medium formulation and operating conditions for organisms, antibiotic production from these genes can be improved. The use of new carbon and nitrogen sources can lead to better microbial growth and antibiotics production (Ilić *et al.*, 2010). The regulation mechanisms of the production of secondary metabolites are not yet fully understood, whereas secondary metabolites must confer an adaptive advantage, the roles of most are not fully understood. This is true even for antibiotics (Bibb, 2005).

The antibiotics may be useful unaltered or they may act as "lead" compounds. These leads can be structurally altered *via* combinatorial chemistry approaches to generate agents with improved activity or reduced toxicity. The growth of secondary metabolite-extracted drugs bears important challenges in multiple areas. The prime challenge is the supply of the drug in sufficient quantities to permit preclinical, and hopefully clinical, development, and eventually, if given a successful, clinical consequence, commercial production. Formulation considered as the major concern. A prime requirement for administration of the drug to human patients especially drugs used through the intravenous route is solubility and the natural products generally are highly insoluble in aqueous media (Cragg and Newman, 2005). Figure 1.3 is a summary of the possible outcomes of a typical bioactivity guided fractionation of any crude extract from natural source and the schematic representation shows that at in any stage would lead to a wide array of possible outcomes. Furthermore, these outcomes might provide unexpected opportunities for modifying the discovery design during subsequent stages.

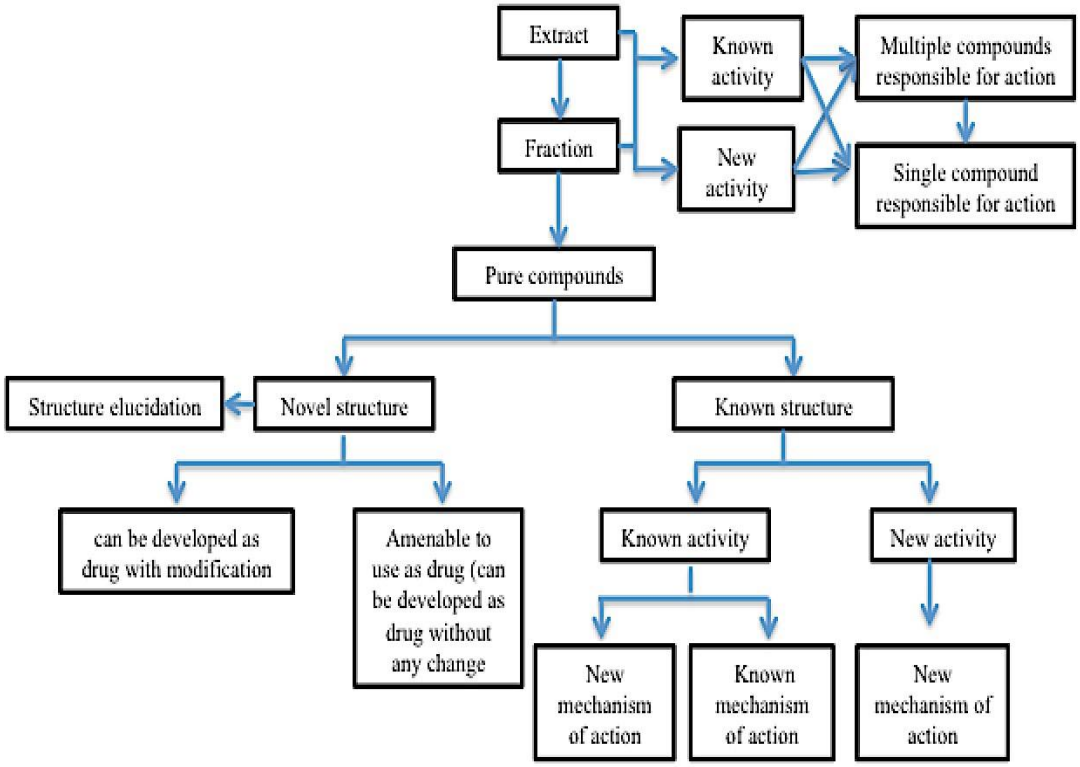


Figure 1.3: Stages of natural products discovery

Modified from (Katiyar *et al*., 2012).

1.9 Organisms that produce antibiotics

Brief history of antibiotics and producer organisms

In 1684 Antonie van Leeuwenhoek was the first person to discover the existence of bacteria by grounding small spherical lenses to observe unicellular bacteria. Microbiology did not advance until 200 years later by pioneers such as Louis Pasteur, Robert Koch and Ferdinand Cohn. The discovery of the first antibiotic penicillin from the mold *Penicillium notatum* by Sir Alexander Fleming opened up a completely new era of chemotherapy, thereby changing the quality of human life (Silber *et al*., 2016).

The first to describe a strain of *Streptomyces* was Ferdinand Cohn, this name was eventually conferred by Selman Waksman in 1942. Waksman's interest in *Streptomyces* lead to the discovery that *Streptomyces* spp. produced antibiotics; he

was awarded with the Nobel Prize for his work. Antibiotics derived from *Streptomyces* spp. began with streptothricin and, two years later, streptomycin. Streptomycin was used commercially and with success (Chater, 2006). The importance of these microorganisms can be seen in the list of antibiotics and their dates of discovery shown in Table 1.2. Antibiotics sourced from the genus *Streptomyces* and other actinomycetes were of paramount importance in their development (de Lima Procópio *et al.*, 2012).

Table 1.2: Key findings and dates of antibiotics.

Antibiotic	Year of discovery	Year of introduction	Year resistance detected	Producing organism
Penicillin	1928	1938	1965	*Penicillium notatum*
Streptomycin	1943	1944	1948	*S. griseus*
Cephalosporin	1945	1964	Unclear	*S. clavuligerus*
Bacitracin	1945	Unclear	Unclear	*B. licheniformis*
Polymyxin	1947	Unclear	Unclear	*B. polymyxa*
Chloramphenicol	1946	1948	1955	*S. venezuelae*
Neomycin	1949	Unclear	Unclear	*S. fradiae*
Chlortetracycline	1944	1952	1959	*S. aureofaciens*
Nystatin	1950	Unclear	Unclear	*S. noursei*
Erythromycin	1948	1951	1955	*S. erythreus*
Fidaxomicin	1948	2011	1977	*Dactylosporangium aurantiacum*
Viomycin	1951	Unclear	Unclear	*S. vinaceus, S. capreolus*
Virginiamycin	1952	Unclear	Unclear	*S. pristinaespiralis, S. virginiae*
Lincomycin	1952	Unclear	Unclear	*S. lincolnensis*
Cycloserine	1955	2000	2000	*S. garyphalus*
Vancomycin	1956	1972	1988	*S. orientalis*
Noviobiocin	1956	Unclear	Unclear	*S. niveus*
Rifamycin	1957	1985	1962	*Amycolatopsis mediterranei*
Kanamycin	1957	Unclear	Unclear	*S. kanamyceticus*

Antibiotic	Year of discovery	Year of introduction	Year resistance detected	Producing organism
Fusidic acid	1963	Unclear	Unclear	*Fusidium coccineum*
Gentamicin	1963	1967	1979	*Micromonospora purpurea*
Fosfomycin	1969	Unclear	Unclear	*S. fradiae*
Ribostamycin	1970	Unclear	Unclear	*S. ribosidificus*
Mupirocin	1985	Unclear	Unclear	*Pseudomonas fluorescens*
Daptomycin	1986	2003	1987	*S. roseosporus*
Platensimycin	2006	Unclear	Unclear	*S. platensis*

Modified from (de Lima Procópio *et al*., 2012, Lewis, 2013, Hede, 2014)
Note: The year of discovery, introduction and resistance detected of the antibiotics in Table 1.2 obtained from de Lima Procópio *et al*., 2012, Lewis, 2013, Hede, 2014) "Unclear" different sources quote different years for discovery and observation of resistance.

Microorganisms have been shown to produce abundant metabolites with different activities including antimicrobial, anticancer and antiviral (Tawiah *et al*., 2012) Research carried out on soil bacteria and fungi have revealed that microorganisms are a rich source of structurally distinctive bioactive substances (Fenical, 1993). After the discovery of penicillin, various drugs were discovered as a result of studying microorganisms. As mentioned earlier, several of the pathogens (e.g., *Mycobacterium tuberculosis, Staphylococcus aureus*) involved in infectious disease are rapidly increasing resistance to the currently available antibiotics, thus rendering the treatment of such infections very challenging (Bhavnani and Ballow, 2000), and has caused research progress around the world to put a priority on antibiotic development, hence the urgent need develop more effective antibiotics from bacteria and fungi. Until recently, most of antimicrobial compounds were isolated from terrestrial microorganisms (Tawiah *et al*., 2012). Microbial sources are responsible for the production of thousands of natural antibiotics with more than 50% produced by actinomycetes, 10% - 15% produced by non-filamentous bacteria and approximately 20% by filamentous fungi. In the past decade, there has been increased interest in fungi for the progress of anti-infective lead compounds, increasing the awareness of their importance in biotechnology (Silber *et al*., 2016)**.**

1.9.1 Actinomycetes

Recent reports show that the actinomycete group of microorganisms still remains an important source of antibiotics (El-Naggar *et al*., 2013). Indeed, the growing economic importance of this group of actinomycetes is due to the fact that the majority of antibiotic producing streptomycetes are found among this group (Suneetha and Raj, 2011). *Streptomyces*, a common genus in the actinomycetes, has long been of interest due to its diversity of chemical constituents and biological activities.

Actinomycetes are Gram-positive bacteria belonging to the order Actinomycetales, characterized by the formation of substrate and aerial mycelium on solid media, presence of spores and a high G+C content of the DNA 60-70 mol % (Shobha and Onkarappa, 2010). Actinomycetes are one of the most attractive sources of novel antibiotics (Lazzarini *et al*., 2000, Attimarad *et al*., 2012)**.** Researchers have so far discovered biologically active compounds of microbial origin, around 23, 000 bioactive secondary metabolites produced by microorganisms have been reported and of which over 10, 000 are produced by actinomycetes. Among actinomycetes, approx.7,600 compounds are produced by *Streptomyces* species (Prashith Kekuda *et al*., 2010, Subramani and Aalbersberg, 2012). Several well-known antibiotics have been isolated from this genus, for example, chloramphenicol, streptomycin, lincomycin, tetracycline (Intaraudom *et al*., 2011) , thienamycin, cephamycin C, penicillin N, as well as drugs with anticancer, antifungal, and immunosuppressant activities (Sudha and Masilamani, 2012), which has made streptomycetes the primary antibiotic-producing organisms exploited by the pharmaceutical industry.

The metabolic diversity of the *Actinomycetes* family is due to their large genome, which has hundreds of transcription factors that control gene expression allowing them to respond to their specific needs (Goshi *et al*., 2002, Saadoun *et al*., 2007a). Accordingly, screening of actinomycetes for potential drugs is still a matter of concern for medical community of many research centers (Jongrungruangchok *et al*., 2006) and they are still one of the most investigated groups particularly members of the genus *Streptomyces* (Boudjella *et al*., 2006). The number of antimicrobial compounds reported from the species of this genus per year has increased almost exponentially for several decades. As a result of the increasing prevalence of antibiotic-resistant pathogens and the pharmacological limitations of antibiotics, there

is a requirement for new antimicrobial substances. With the seemingly exponential emergence of microorganisms becoming resistant to the clinically available antibiotics already marketed, the need for discovering novel drugs is urgent (Deepa *et al*., 2012).

Among various genera, *Streptomyces*, *Saccharopolyspora*, *Amycolatopsis*, *Micromonospora* and *Actinoplanes* are the major producers of commercially important biomolecules. As the streptomycetes are very potent producers of secondary metabolites (Atta *et al*., 2012, Subramani and Aalbersberg, 2012, Kekuda and Shobha, 2010) these will be looked at in detail.

The genus *Streptomyces*

Streptomycetes are aerobic, Gram-positive, filamentous bacteria that undergo morphological differentiation during their life cycle. Spores usually occur but when nutrients and moisture are present, germination can occur and progress to vegetative mycelium with environmental changes such as depletion of nutrients and dehydration, differential processes are set into motion; desiccation and starvation resistant spores are formed again. Production of pigments, antibiotics and other secondary metabolites is initiated at the same time (Maheshwari *et al*., 2010).

Streptomycetes produce extensive branching vegetative (substrate) mycelium and aerial mycelium bearing chains of arthrospores. The substrate mycelium and spores can be pigmented, but also diffusible pigments are produced (Bentley *et al*., 2002, Nayaka and Babu, 2014). On agar plates, they form lichenoid, leathery or butyrous colonies. The cell wall of streptomycetes contains LL-diaminopimelic acid and glycine (Kumar *et al*., 2012a).

Carbon sources from a wide range of organic compounds are utilized by streptomycetes, such as cellulose and lignin. Inorganic nitrogen source can also be utilized (Arunachalam *et al*., 2010).

Life Cycle

Streptomycetes resemble fungi in their elaborate life cycle, in addition to their cellular structure (Figure 1.4). The development stage of streptomycete during vegetative growth causes DNA replication without division of cells. Filamentous structures are created during this cycle. Post vegetative growth, streptomycetes

reproduce and disperse through the formation of spores, called conidia. Spores in aerial filaments called sporophores are produced which rise above the colony. The resemblance to multicellular eukaryotes that streptomycetes have enables researchers to utilise a simpler system to study their development and life cycle (Panda and Rameshaiah, 2009).

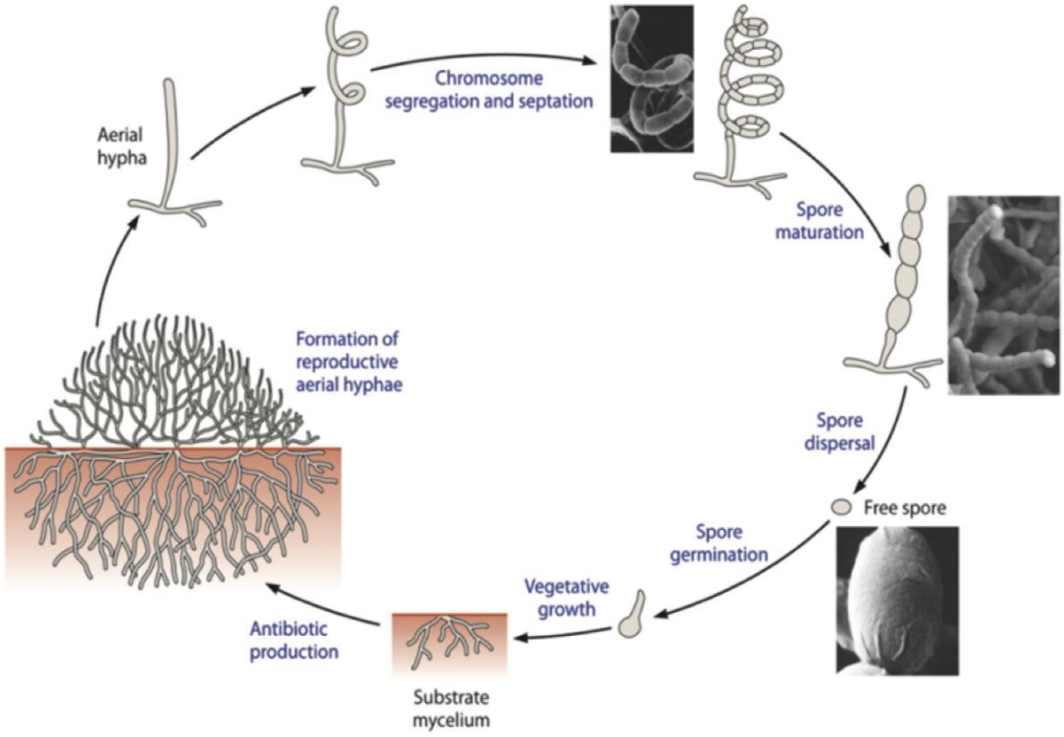


Figure 1.4: Life cycle of *Streptomyces*

Modified from (Wu, 2016)

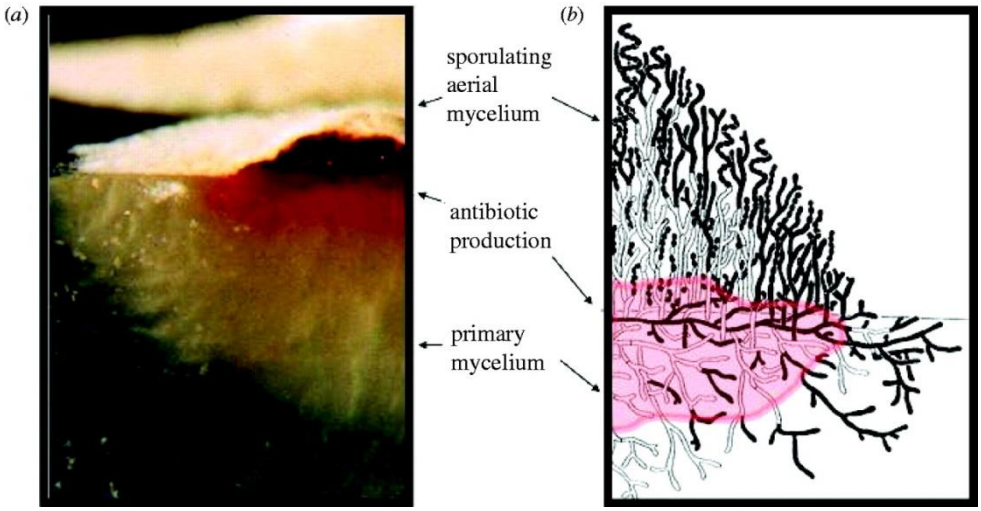


Figure 1.5: (*a*), Vertical sections through a *Streptomyces* colony. Photograph of colony growing on agar. (*b*) A diagram to shows dead cells (white vegetative mycelium) are autolytically degraded to provide the necessary nutrients, this allows the formation of aerial growth and sporulation. Living cells are shown as black (Chater, 2006).

Classification

In general, the taxonomic classification of streptomycetes is based on morphological and biochemical characteristics (Gherbawy *et al.*, 2012, Haritha *et al*., 2012). Physiological tests such as preforming the growth at different temperature range from 4 to 65, PH range from 5.0 to 10.5 and the growth under anaerobic condition (Goodfellow *et al.*, 1987, Kämpfer *et al.*, 1991, Reddy *et al*., 2011, Haritha *et al.*, 2012, Parthasarathi *et al.*, 2012), serological methods (Cross and Spooner, 1963), phage typing (Korn-Wendisch and Schneider, 1992) and protein profiling (Ochi, 1995, Takeuchi *et al.*, 1996) have also been used in their classification. More recently, the application of genetic methods, such as DNA-DNA reassociation (Labeda and Lyons, 1992) and 16S rRNA gene sequence analysis (Kataoka *et al*., 1997, Chen *et al*., 2012, Parthasarathi *et al.*, 2012) has been used. Anderson and Wellington, (2001) review and summarize all of the approaches to clarify the taxonomy of streptomycetes. Multilocus sequence analysis (MLSA) has shown promising potential for refining *Streptomyces* systematics, MLSA using three or four of the genes also shows good resolution for distinguishing between most close strains

and more suitable for discriminating strains that show >99% 16S rRNA gene sequence similarity (Rong and Ying, 2010).

Identification

Within the actinomycetes, the largest number of varieties and species found in natural environments are in the genus *Streptomyces*, their morphology, biology and biochemical activities have been observed to differ greatly. Isolation and description of numerous new species have resulted from this focused research, paving the way for the discovery of new divisions into subgenera or series. Some authors have considered making certain important characters such as morphology, the structure of the substrate mycelium, the nature and formation of aerial mycelium, and branching of pseudohyphae containing spores, and the spore surface in an attempt to make such separations. Cultural criteria, such as growth on different media, colour of aerial and substrate mycelia, and formation of soluble pigments were also taken into account. For the classification of *Streptomyces* spp. certain biochemical criteria such as the utilisation of carbon sources, proteolytic properties, usage of nitrogenous compounds, presence of oxidases and reductases as well as sensitivity to antibiotics and phages, serological reactions, ecological properties and genetic relationships have been used. (Taddei *et al*., 2006a). Based on 41 characteristics, a probabilistic identification matrix for *Streptomyces* spp. identification, characters such as spore chain and spore morphology, pigmentation, physiological abilities, antibiosis and resistance to antibiotics, was developed by (Williams *et al*., 1983). This scheme was applied to unknown isolates, 80 % of which could be assigned to a cluster. (Kämpfer *et al*., 1991) supplemented the method with rapid enzyme tests using fluorogenic substrates. Phage sensitivity and serological tests as well as cellular fatty acid characterisation can be useful for assigning an unknown isolate to the genus *Streptomyces*, the difficulties imposed with species discrimination at the intragenus level were quickly observed (Anderson and Wellington, 2001).

DNA based molecular methods have been used for species differentiation and the identification of *Streptomycetes*. DNA-DNA reassociation is a method measuring the DNA relatedness of two organisms and has proved to be suitable for the investigation of relationships between closely related taxa, such as species. Strains belonging to the same species will generally have greater than 70 % DNA-DNA

relatedness (Anderson and Wellington, 2001, Stackebrandt and Goebel, 1994, Bouchek-Mechiche *et al.*, 2000). While DNA-DNA reassociation has shown to be useful in the identification of *Streptomyces* spp., it should not be used alone, but in connection with other tests, because there is considerable genetic instability of certain regions within the chromosome (Redenbach *et al*., 1993).

Determination of difference in the sequence of the 16S rRNA gene is now a well established standard method for the identification and phylogenetic classification of prokaryotic species, genera and families, but more recently it has also become important as a means to identify an unknown bacterium to the genus or species level (Arunachalam *et al.*, 2010).

The DNA sequences of the ribosomal genes are highly conserved, but the genes also contain variable regions, which sometimes can be useful for species discrimination and Kataoka *et al.*, (1997) utilised the hypervariable region of the 16S rRNA gene to create an index for *Streptomyces* spp. identification. Also repetitive intergenic DNA sequences (rep-PCR) (Sadowsky *et al.*, 1996) and PCR-RFLP of the 65-kDa heat shock protein gene (Steingrube *et al.*, 1997) have been used for the classification and identification of pathogenic and other clinically important *Streptomyces* spp.

1.9.2 Fungi

It is estimated that there are 1.5 million fungal species on earth, of which only about 70 000 have been described so far (Dayalan *et al*., 2011)

Fungal natural products consist of a broad range of compounds. Some of these compounds are medically attractive as drugs and drug leads (Anyaogu and Mortensen, 2015)

Although actinomycetes are second to fungi as sources of secondary metabolites, the fungi produce only about 20% of products with antimicrobial activity. Fungi have long been known to produce antimicrobial agents (Broadbent, 1966).

Classically, *Penicillium chrysogenum* (previously known as *Penicillium notatum*) was the first antibiotic producing organism to be discovered and penicillins, together with cephalosporins produced by *Acremonium chrysogenum* have been one of the mainstays of antibacterial chemotherapy (Muniz *et al*., 2007). Continued research into chemical modifications has lead to several generations of compounds with improved spectra of activity and/or chemical properties. More recently attention has been given

to fungi as sources of novel compounds. For example novel classes of antimicrobial agents (new aromatic polyketides and diketopiperazines) were produced by *Penicillium* spp. from terrestrial and marine sources from Australia (Capon *et al*., 2007), antimicrobial agents (methyl 6-acetyl-4- methoxy-5, 7, 8-trihydroxynaphthalene-2-carboxylate, methyl 6-acetyl-4-methoxy-7,8-dihydroxynaphthalene-2-carboxylate and methyl 6-acetyl-4-methoxy-5, 8-dihydroxynaphthalene-2-carboxylate) have been obtained from Brazilian soil (Petit *et al*., 2009), and from soil in Northern Iran (Gharaei-Fathabad *et al*., 2009), from endophytic fungi (Selim, 2012) and even from filamentous fungi from culture collections (Rančić *et al*., 2006) .

The genus *Penicillium*

Penicillium is one of the most common fungal genera of the micro-fungi group. It has been well over 200 years since the name *Penicillium* was introduced by Link (1809). The definition of Penicillium is 'brush' and it was given this name due to its brush-like appearance under the microscope.
Link defined the three common species *P. candidum, P. glaucum* and *P. expansum*. Thereafter over 1000 names were introduced in the genus, many of which are currently not identifiable due to lack of information as set by modern standards. Some of the other names are considered to have been published invalidly, or are currently recognized to be synonyms of other species (Visagie *et al*., 2014). *Penicillium* spp. are ubiquitous soil fungi that prefer cool and temperate climates and can be found wherever organic material is present. *Penicillium* spp. conidia can be found in the air and dust of indoor environments including houses and public spaces and in and around foods, leather and fabric materials. Some species have a distinctive blue colour due to pigmentation of the conidia causing the blue fuzzy texture on foods such as breads (Ozdemir, 2015). *Penicillium* rots refers to the green and blue mould found on foodstuffs which is caused by different species of *Penicillium*. These particular species are responsible for the post-harvest loss of foods including during transit, storage and post-sales (Agrios, 2005)
With regards to human diseases, *Penicillium purpurogenum*, is a soil and plant contaminant that can grow at 37°C has been involved in only three human cases of pulmonary infection in patients afflicted by haematological diseases but no infections

in animal have been reported. Approximately 15 various species of *Penicillium* are responsible for mycotic infection in immunocompromised patient. (Zanatta *et al*., 2006)

The genus *Penicillium* became well established after the discovery 'penicillin' from a culture of *P. notatum* by Alexander Fleming and his studies as it related to growth inhibition of the pathogenic bacterium *Staphylococcus aureus* (Raper and Thom, 1949) .

Vegetative growth of *Penicillium* spp is in the form of mycelium which grows and branches through the growth medium. In response to starvation, aerial hyphae (conidiophores) are formed on which the asexual spores (conidia) develop. This may branch but at the tip develops finger-like phialides from which the conidia develop (Figure 1.6, Visagie *et al*., 2014).

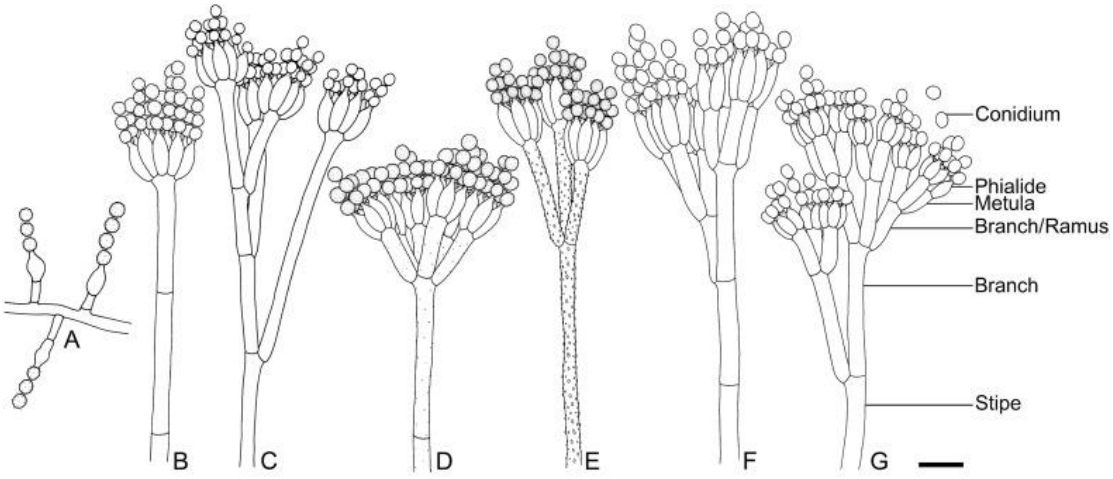


Figure 1.6: Conidiophore branching patterns seen in *Penicillium* spp. A. Conidiophores with solitary phialides. B. Monoverticillate. C. Divaricate. D, E. Biverticillate. F. Terverticillate. G. Quaterverticillate from (Visagie *et al*., 2014)

The genus *Penicillium* has been shown to produce a wide range of compounds with a broad variety of biological activities (Debbab *et al*., 2010, Nakashima *et al*., 2008), antibacterial such as negapillin, antimicrobial agent isolated from metabolites of *Penicillium chrysogenum* (Shimi *et al*., 1966), antifungal such as atpenin isolated from *Penicillium* sp. FO-125 by Omura *et* al., (1988), and immunosuppressive agent such as stevastelin A, B and B3 isolated from *Penicillium* sp. by Morino *et al*., (1996).

Kurobane *et al*., (1981) showed that *Penicillium brefeldianum* produced fulvic acid which has antiviral, antifungal, antioxidant and antibiotic activities. (Maskey *et al*., 2003) isolated two active substances 8-O-methylaverufin and 1,8-O-dimethylaverantin as novel antifungal agents from *Penicillium chrysogenum*. (Nam *et al*., 2000) established that compounds 8-O- methylsclererotiorinamine isolated from *Penicillium multicolor* expressed antimicrobial activity. Funiculosin isolated from *Penicillium funiculosum* also has antivirus activity against Newcastle disease virus (NDV), Miyadera strain (Ando *et al*., 1969).

Classification and identification of *Penicillium*

Penicillium spp belongs to an ascomycete of the family *Eurotiaceae* and kingdom of fungi, the genus, *Penicillium*, is ubiquitous and most of the species are saprophytes (Talbot, 1971). One of its key roles in nature is the disintegration of organic materials and the species cause overwhelming rots as pre- and postharvest pathogens on food harvests and also produces varied forms of mycotoxins. However some species when exploited have a positive impact on the production of certain foods including cheese such as Camembert or Roquefort and fermented sausages (Visagie *et al*., 2014). Selected strains of these fungi are currently used as starter cultures as a way to colonize homogeneously the cheese or meat piece, therefore inhibiting the ultimate colonization by unwanted fungi and bacteria. Some of the fungal species include *Penicillium nalgiovense* and *Penicillium chrysogenum* for meat products, *Penicillium roqueforti* for Roquefort cheese, and *Penicillium camemberti* for Camembert cheese (Laich *et al*., 2002).

Traditionally the identification and classification of fungi was based upon analysis of macroscopic and microscopic structures (morphology) (Larone, 1993). Physiological and biochemical procedures, applied in combination with morphology, (Pitt, 1995) and their features; may vary significantly depending on the environment and the conditions they are exposed to. More recently, molecular techniques have been used to assist in distinguishing the microorganisms (Jimenez *et al*., 1999). Ribosomal DNA (rDNA) is a useful aid to fungal phylogeny studies as it encodes the highly polymorphic 600-800 bp ITS region (Internal Transcribed Spacers), which is used for the isolation of many fungal species (Anderson and Cairney, 2004). This region has

recently been accepted as the official sequence for identification of fungi (Schoch *et al*., 2012)

1.10 Sources of Antibiotic- producing organisms

1.10.1 Soil

The significance of terrestrial bacteria and fungi as foundations of valuable bioactive compounds has been well recognized for over half a century. As a result, over 120 of the most crucial medicines used (penicillins, cyclosporine A, adriamycin, etc.) are acquired from terrestrial (soil) microorganisms (Karna Radjasa, 2004). Soil is a widely explored ecological niche for microorganisms which yield significant biologically active natural products including antibiotics and used in industrial antibiotic production (Kaur *et al*., 2014, Kumar *et al*., 2010)**.** Some of the antibiotic drugs have been derived from microorganisms that inhabit the soil; this includes fungi (20% of isolated antibiotics), actinomycetes (70%) and eubacteria (10%) (Makut and Owolewa, 2011).

The most common habitat for streptomycetes is soil, this bacteria can be found in various types of soils (Katsifas *et al*., 2000, Wu *et al.*, 2007). Surfaces such as plant residue and fungal hyphae have been observed as good habitats for streptomycetes colonization. This may be important in the degradation of litter in soil (Maheshwari *et al*., 2010).

Streptomycetes may play a role in promoting plant growth, through control of root pathogens or in some indirect way, since some species are able to produce antifungal compounds (Yuan and Crawford, 1995). Streptomycetes can be used as biocontrol agents (Kanini *et al.*, 2013). On the other hand, potato scab, a plant disease, is caused by several species of *Streptomyces*, which causes significant financial losses to the agricultural community (Takeuchi *et al*., 1996, Lorang *et al.*, 1995).

However, soil from many parts of the world has been very extensively studied and isolation of new organisms and new active compounds is therefore difficult. Because of this attention has been turned to neglected sources in the hope of finding new strains of organisms producing novel metabolites. For example, marine organisms have become vital sources of novel products exhibiting antimicrobial, antiviral, antitumor and cardio-active properties (Kokare *et al*., 2004, Saadoun *et al.*, 2007b)

which may serve as model systems in the discovery of new drugs. Although soils have been screened by the pharmaceutical industry for the past five decades, only a small fraction of actinomycetes have been discovered (Atta *et al.*, 2012). Actinomycetes from unexplored habitats have gained considerable attention in recent years for the production of bioactive metabolites. The list of novel actinomycetes and products derived from poorly explored areas of the world stresses the importance of investigating new habitats (Usha Kiranmayi *et al.*, 2011).

1.10.2 Aquatic sources

Streptomycetes can be found in nearly all aquatic environments, fresh water as well as marine environments (Gebreyohannes *et al.*, 2013). This does not however, mean they are actively growing in aquatic environments, Streptomyces may be washed into marine environments from terrestrial sources e.g. by exporting spores that are hardy into the water which may remain *in situ* for extended periods of time (Zaitlin and Watson, 2006). However steptomycetes may be normal components of the aquatic biota. For example, streptomycetes accounted for 2-5 % of the microbial community, and were found to be an indigenous population in coastal marsh sediment (Moran *et al.*, 1995).

Earthy odours have been associated with actinomycetes, but it is not known whether actinomycetes are a contributing factor to earthy odours in freshwater. The earthy smelling secondary metabolites geosmin and 2-methylisoborneol (MIB) were identified from actinomycete cultures in the late 1960s. Since then actinomycete derived taste and odour in drinking water have meant that actinomycetes have gained considerable notoriety in the water supply industry (Zaitlin and Watson, 2006). It is noteworthy to mention here that *Streptomyces* spp. have also been isolated from severely cold environments like Antarctica (Kim *et al.*, 2007).

The aquatic environment is now being recognized as an abundant and untouched source of different natural products. The marine environment in and of itself contains organisms with diverse taxonomic that show distinctive physiological and structural characteristics that allow them to survive even that harshest of environmental conditions, with the potential production of new secondary metabolites that have not been found in terrestrial microorganisms (Tawiah *et al.*, 2012).

Bacteria and fungi are being isolated from various marine sources, including invertebrates, sediment and algae. These microbes have been examined for the discovery of new bioactive compounds; sponges have been recognized by chemists as an interesting source of bioactive compounds, Holler *et al*., 2000 isolated 681 fungal strains from 16 species of sponges from six different locations, the isolated were belonged to 13 genera of *Ascomycota*, 2 of *Zygomycota*, and 37 of mitosporic fungi. For the biological activity just ninety-two of the 681 isolates were selected for screening, the result showed that 75 strains of 92 (81.5%) were active in at least one agar diffusion assay for antibacterial, antifungal or antialgal activity, on other hand they found only six strains (7%) were active against the Gram-negative bacterium *E. coli*, while 33 strains (36%) were active against the Gram-positive bacterium, *B. megaterium*. Fungi were isolated from sponges from varied climatic and geographical marine habitats Holler *et al*., 2000. Marine fungi are recognized as a rich source of bioactive compounds with potential biomedical and pharmaceutical applications (Shaala and Youssef, 2015).The microbial biodiversity of marine environments is vast, and it is yet to be explored and characterized in both taxonomically and chemically. Therefore, secondary metabolites that are entirely created by marine organisms are assumed to facilitate their adaptation and survival in the marine environments categorized by very special conditions. A new compound terretrione D **2** and known compound terretrione C **6** were isolated from marine-derived fungus *Penicillium* sp. CYE-87 showed significant antimigratory activity against the highly metastatic triple-negative human breast cancer cells MDA-MB-231 with IC_{50} values of 16.5 and 17.6 μM, respectively. Also, these compounds showed reasonable activity against *C. albicans* with a MIC value of 32 μg/mL (Silber *et al*., 2016).

1.10.3 Insects

The existence of actinomycetes has been reported in association with insect habitats. The two best described examples being those of the Attine ants (*Atta sp*.), known to cultivate fungal gardens as a food resource and which appear to have a symbiosis with a *Pseudonocardia* bacterial species (residing on the ants' integuments) that assists in defending the fungus from a parasite through the production of secondary metabolites (not characterized) (Mueller *et al.,* 2008) and secondly the beewolf wasp (*Philanthus triangulum*) which has specialised antennae containing a *Streptomyces* sp. which is

thought to produce antibiotics (not yet characterized) that the wasp smears over brood cells prior to oviposition to protect eggs from a fungal parasite (Kaltenpoth *et al.*, 2005).

Aruna *et al*., 2008 reported antagonistic activity (not characterized) of new strain *Streptomyces tritolerans* sp. isolated from earthworm gut. The results showed that earthworm gut is favorable to streptomycetes and can be a vital habitat for adopted bacteria possessing antimicrobial activity. They should receive concentrated attention in research for biological controls worldwide (Aruna *et al*., 2008).

(Ebrahimipour *et al*., 2011) reported the antagonistic activity (again not characterized) of a bacterium isolated from the digestive system of Iranian endemic slug, *Parmacella iberica,* and gave details of its biochemical identification and phylogenetic relationships and extraction of antimicrobial substance.

Until recently, the majority of antimicrobial compounds were isolated from terrestrial microorganisms. In the last two decades however, the rate of discovery of novel compounds from this source has declined significantly. Recently researchers have begun investigating unconventional sources due to lack of research within those particular areas. This includes, on terrestrial invertebrates (Madden *et al*., 2013).

Fungi isolated from marine invertebrates are of substantial significance as novel promising sources of distinctive secondary metabolites with important biomedical potential (Yue *et al*., 2015)

Modern researchers highlight on the need to study unique unexplored places like rain forests, marine sponges, mangroves and endophytes for pharmacologically active compounds. The ever growing list of fresh microorganisms and the products resulting from poorly explored areas of the world like Australia, Jordan and Antarctica shows that new habitats if explored carefully can bring great benefit. The biopharmaceutical industry has become increasingly interested in new antibiotics to tackle the challenge of resistance (Kumar *et al*., 2010).

1.11 AIMS OF THE STUDY

Marine invertebrates have been shown to be sources of antibiotic producing microorganisms but, apart from social insects, terrestrial invertebrates and freshwater invertebrates in particular have been relatively little studied. The aim of this project was therefore to screen invertebrates primarily for novel actinomycetes, which may in turn possess novel biosynthetic capabilities but also including other antibiotic producing organisms e.g. fungi.

The main objectives of the programme of study were:

1. To field collect aquatic and terrestrial invertebrates and investigate them for the presence of actinomycetes and other organism by culture on selective media.

2. To identify isolates that showed antibiotic activity by primary screening against Gram-positive and Gram-negative bacteria.

3. To use PCR amplification of 16S/18S rDNA, to profile species associated with different sources. Sequencing of ITS (internal transcribed spacer) regions would also be required for the identification of any fungi isolated.

4. To determine antimicrobial activity of isolated strains by screening isolates for inhibitory activity against a range of test organisms.

5. To purify the active compounds from species showing good activity using HPLC, determine their chemical structures and test their activity against antibiotic resistant pathogens and tissue cultures of cancer cells.

Chapter 2

2. MATERIALS AND METHODS

2.1 Sample of the invertebrates

Some of the invertebrate samples were obtained from Commercial companies and the rest were provided by the School of Environment and Life Sciences of the University of Salford. The origins and numbers collected are shown in Table 2.1 and 2.2.

Table 2.1: The aquatic invertebrates and the origins they were collected from.

Aquatic invertebrates			**Origin**
Latin name	Common name	Number	
Gammarus pulex	Freshwater shrimp	60-70	Collected from River Tame
Daphnia sp.	Water flea	100-150	Blades Biological Ltd (Collected from Kent)
Chironomus sp. larva	Bloodworm	15-20	Sciento (Collected from Manchester area)
Eropobdella octoculata	A leech	10-15	Sciento (Collected from Manchester area)
Lumbriculus variegatus	Black worm	*30*-40	Sciento (Collected from Manchester area)
Nereis diversicolor	Bristle worm	4-5	Sciento (Collected from Formby area)
Actinia equine	Sea anemone	5-10	Collected from Seaton Carew, Hartlepool
Baetidae sp. nymph	Swimming mayfly nymph	15-20	Collected from River Tame
Baetidae sp. nymph	Flattened mayfly nymph	15-20	Collected from River Tame
Gyrinus sp. larva	Whirligig beetle Larva	15-20	Collected from River Tame

Aquatic invertebrates			**Origin**
Latin name	Common name	Number	
Tubifex tubifex larva	Sludge worm	20-25	Collected from River Tame
Culicoides sp. larva	Biting midge larva	15-20	Collected from River Tame
Dendrocoelum lacteum	Flatworm	15-20	Sciento (Collected from Whitefield area of Manchester)
Polycelis nigra	Flatworm	15-20	Sciento (Collected from Whitefield area of Manchester)
Crangonyx pseudogracilis	Northern river crangonyctid	40-50	Sciento (Collected from Whitefield area of Manchester)
Asellus aquaticus	Waterlouse	15-20	Sciento (Collected from Whitefield area of Manchester)
Asellus aquaticus	Waterlouse	15-20	Collected from River Tame
Planorbis planorbis	Ram's horn snail	5-10	Collected from Manchester area
Lymnaea stagnalis	Great pond Snail	5-10	Collected from Manchester area
Planorbis corneus	Great Ram's-horn snail	5-10	Collected from Manchester area
Calopteryx sp.	Damselfly nymphs	15-20	Blades Biological Ltd (Collected from Kent)

Table 2.2: The terrestrial invertebrates and the origins they were collected from.

Terrestrial invertebrates			**Origin**
Latin name	Common name	Number	
Helix pomatia	Roman snail	30-40	Collected from Manchester area
Tenebrio molitor (larva, pupa and adult)	Yellow mealworm	20-30	Sciento and Livefood.co.uk (Commercially bred cultures)
Arion ater	Black slug	10-15	Collected from a North Cheshire field
Myrmica rubra	Red ant	70-80	Blades Biological Ltd (Collected from Kent)
Lasius Flavus	Yellow meadow ant	70-80	Blades Biological Ltd (Collected from Kent)
Psyllobora 22-punctata	22-spot Ladybird	5-10	Collected from Manchester area
Oniscus asellus	Common shiny woodlouse	5-10	Collected from Westhoughton area
Helix aspersa	Garden snail	5-10	Sciento (Collected from Whitefield area of Manchester)
Vespula vulgaris	Common wasp	15-20	Collected from a House in Rainhill, Merseyside
Eisenia fetida	Tiger worm	5-10	Blades Biological Ltd (Collected from Kent)
Tribolium castaneum	Red flour beetle	*15-20*	Blades Biological Ltd (Breeding)
Lasius niger	Black ants	30-40	Blades Biological Ltd (Collected from Kent)
Lumbricus terrestris	Common Earthworm	5-10	Blades Biological Ltd (Breeding)

Terrestrial invertebrates			Origin
Latin name	Common name	Number	
Eisenia andrei	Red tiger worm	5-10	Blades Biological Ltd (Collected from Kent)

2.2 Test organisms

The test organisms were obtained from the culture collection of the Microbiology Laboratory at the School of Environment and Life Sciences, University of Salford. All test organisms were incubated at 37ºC for 24 h except for *P. fluorescens* and *S. cerevisiae* which were incubated at 28±2 ºC for 24 h. The organisms and original sources are given below.

2.2.1. Gram-positive Bacteria

Bacillus cereus ATCC 10876 (Fakruddin *et al*., 2012), *Staphylococcus aureus* ATCC 25923 (Taleb-Contini *et al*., 2003), *Staphylococcus aureus* AOH1 (University stock and unknown source), Methicillin-resistant *Staphylococcus aureus* (MRSA252 obtained from Public Health England) (Holden *et al*., 2004), *Enterococcus faecium* NCTC 7171 (Cushnie *et al*., 2003) and *Enterococcus faecalis* NCIMB 13280 (Thomson *et al*., 2011).

2.2.2. Gram-negative bacteria

Escherichia coli ATCC10536 (Šaric *et al*., 2009), Extended Spectrum Beta-Lactamase (ESBL) producing *E. coli* (obtained from Public Health England), *Pseudomonas aeruginosa* NCTC 15692 (De soyza *et al*., 2013), *Pseudomonas fluorescens* ATCC 13525 (Arora *et al*., 2012) and *Klebsiella pneumoniae* ATCC 10031 (Omoregie, *et al*., 2013).

2.2.3. YEAST

Saccharomyces cerevisiae ATCC 9896 (Christman *et al*., 1956)

2.3 Culture Media Preparation

Muller Hinton Agar (MHA) was used for disk and well diffusion sensitivity tests and Muller Hinton Broth (MHA) (both from Oxoid Ltd, Basingstoke, UK) was used for preparing suspensions of the test organisms and determination of broth dilution minimal inhibitory concentrations (MIC). Nutrient Broth (NB; Oxoid) was used for preparing suspensions of the test organisms, Nutrient Agar (NA; Oxoid) was used for agar overlay technique experiment in the primary screening for antibiotic activity. Tryptone Soya Broth (TSB), Yeast Extract Broth (YEB) (both Oxoid, UK) and Malt Extract Broth (MEB; Fluka obtained from Sigma, UK) were used for comparative antibiotic production in the secondary screening and Actinomycete Isolation Agar (AIA; Fluka obtained from Sigma, UK) was used to isolate antibiotic producing organisms. Media were made up according to the manufacturer's instructions. Actinomycete Isolation Broth (AIB), Starch Broth (SB) with two different nitrogen sources, Glucose Nitrate Broth (GNB) and Starch Broth (SB2) mixed in the ratio 2:1 with Nutrient Broth (NB) before sterilization with two different nitrogen sources were all used for comparative antibiotic production in secondary screening.

All media were autoclaved at 121° for 15 min. Agar containing media were cooled to 50°C before pouring into Petri dishes. After setting, the plates were dried inverted at 50°C for 15-25 min.

AIB was prepared by suspending 68.4 mM glycerol (Sigma, UK) in 1 L of distilled water, 2.48 mM sodium caseinate (Fisher, UK), 0.76 mM L-asparagine (Sigma, UK), 41.64 mM sodium propionate (CH_3CH_2COONa) (Sigma, UK), 2.87026 mM dipotassium phosphate (K_2HPO_4) (Sigma, UK), 0.083 mM magnesium sulphate ($MgSO_4$) (Sigma, UK), 0.0036 mM ferrous sulphate ($FeSO_4.7H_2O$) (Sigma, UK) were added. The mixture was stirred for 15 min. The pH was adjusted to 7±0.2 at 25°C before autoclaving.

SB was prepared by hydrolyzing 58.42 mM of soluble starch (Sigma, UK) in 300 ml of distilled water, with 27.42 mM hydrochloric acid (HCl) (Fisher, UK) in an autoclave at 120ºC for 6 min, cooling, then neutralizing with concentrated sodium hydroxide (NaOH) (Fisher, UK). 23.53 mM sodium nitrate ($NaNO_3$) (Sigma, UK) or 15.13552 mM ammonium sulfate $(NH_4)_2SO_4$ (Sigma, UK), 5.74053 mM K_2HPO_4, 0.20286 mM magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) (Sigma, UK), 6.70679 mM Potassium chloride (KCl) (Sigma, UK), 9.99132 mM Calcium carbonate ($CaCO_3$) (Sigma, UK) and 0.0036 mM $FeSO_4.7H_2O$ were added and the volume was

then made up to 1 L with water. The mixture was stirred for 15 min. The pH was adjusted to 7±0.2 @25°C before autoclaving.

GNB was prepared by suspending 111.015 mM glucose (Fisher, UK) in 1 L of distilled water. $NaNO_3$ 23.53 mM, K_2HPO_4 5.74 mM, 0.203 mM Mg $SO_4.7H_2O$, 6.71 mM KCl, 9.99 mM $CaCO_3$ and 0.0036 mM $FeSO_4.7H_2O$ were added. The mixture was stirred for 15 min. The pH was adjusted to 7±0.2 at 25°C before autoclaving.

2.4 Isolation of microorganisms

Each group of invertebrates was externally sterilized with ethanol 70% for 30 seconds then washed with sterile distilled water. According to the invertebrates size, 5-10 small sized invertebrates (less than 2 cm) or 1-4 invertebrates (between 3 to 5 cm) were put into 50 ml sterile conical end tubes, covered with 1 ml of sterile water then homogenized by grinding with a glass rod. One mililitre of sterile distilled water was then added to reduce the viscosity and serial 10-fold dilutions up to 10^{-5} made in distilled water.

One hundred microliters of each dilution was transferred to a AIA medium and spread evenly on the surface of the medium using a sterile glass spreader and then incubated for 7 days at 28±2ºC. Filamentous colonies of actinomycetes or fungi characterized by their sharp round edges and powdery aspect sinking in the agar plate were picked and streaked on to AIA medium and then incubated at 28 ±2 ºC for 7 d to obtain pure colonies of organisms.

Larger invertebrates (e.g. slugs/snails) used were surface sterilized with 70% ethanol for 30 sec and then washed with sterile distilled water. The digestive tract which was likely to contain the majority of bacteria was removed from each invertebrate and three digestive tracts were put in a 50 ml sterile conical end tube, covered with 1 ml of sterile water, then homogenized by grinding with a glass rod, then plated out and incubated as before and filamentous colonies picked and transferred colonies on to AIA medium.

2.5 Primary screening

2.5.1 Agar overlay technique

Isolated organisms were subjected to primary screening procedures for assessment of their ability to produce antimicrobial activity using an agar overlay assay (Tyc *et al*., 2014). A portion (50 µl) of 24 h culture (NB) of *E. coli* ATCC10536 and *S. aureus* ATCC 25923 was aseptically inoculated into a melted and cooled NA (25 ml at 40-45°C) and the agar was poured onto the AIA plates containing single isolated colonies of isolates which had been incubated for 7 d at 28±2ºC. Then the plates were incubated at 37°C for 24 h. At the end of incubation period the diameter of any clear zone around the isolated colony was measured and recorded in mm.

2.5.2 Streak or single colony pattern on agar plates to detect antimicrobial activity

A single isolate colony was removed and streaked onto AIA, a single streak was made on one side of the agar plate or a single colony inoculate in the middle of the plate and incubated for seven days at 28±2ºC. After 7 d the plates were removed and five test organisms that had been grown overnight on NB (*S. aureus* ATCC 25923, *S aureus* AOH1, *B. cereus* ATCC 10876, *E. coli* ATCC 10536 and *P. fluorescens* ATCC 13525) were streaked on the same agar plate (Figure 2.1). The plates were incubated for 24 h at 28±2 ºC to allow *P. fluorescens* ATCC 13525 growth then incubated for 24 h at 37 °C. The diameters of any clear zones were measured as in 2.5.1

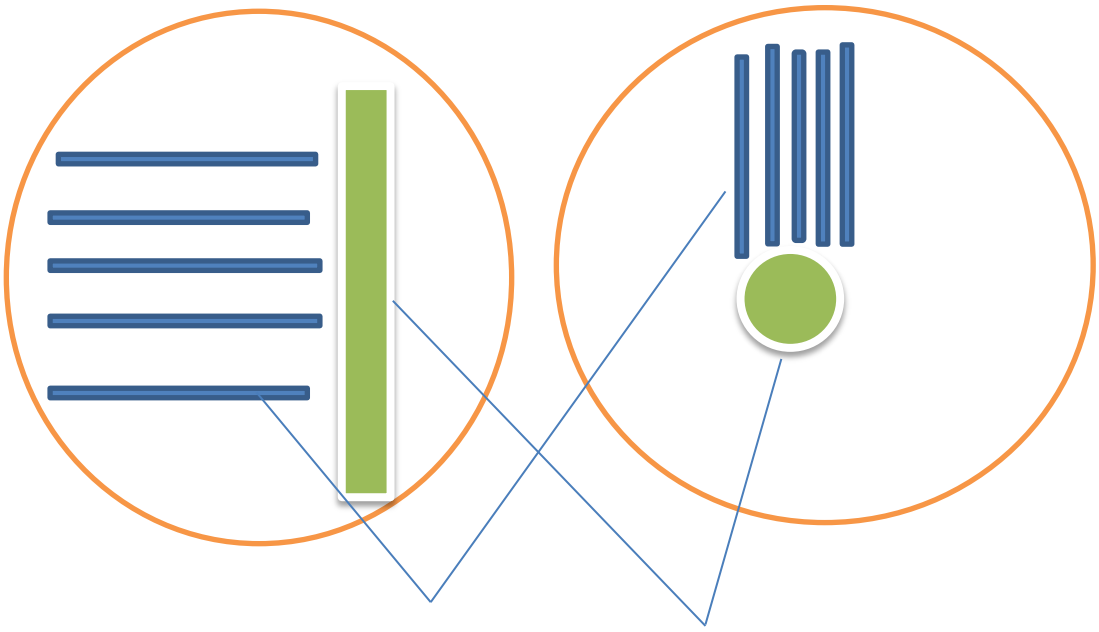


Figure 2.1: Streak patterns on agar plates to detect antimicrobial activity

2.6 Maintenance of cultures

After antimicrobial activity screening, the isolates showing activity were maintained as suspensions of cells and mycelial fragments in aqueous 10% glycerol (v/v) at -18°C.

2.7 Secondary screening (submerged culture)

Comparative antibiotic production using different media

The primary isolates possessing antibacterial activity were subjected to a secondary screening procedure. Nine different media, SNB ($NaNO_3$ as source of nitrogen), SNHB ($(NH_4)_2SO_4$ as source of nitrogen), SNB ($NaNO_3$ as source of nitrogen) mixed with NB 2:1, SNHB ($(NH_4)_2SO_4$ as source of nitrogen) mixed with NB 2:1, GNB, YMB, YEB, TSB and AIB were aliquotted in 50 ml portions in 250 conical flasks, plugged with cotton wool, sterilized and cooled. Flasks were inoculated with two loopfuls of isolate Es1 isolated from *Helix aspersa* (which showed highest antibacterial activity against Gram positive and Gram negative bacteria in the primary screen) and incubated at 28±2 ºC on a rotary shaker at 120 rpm for 7 d. Cultures with good growth were then removed and filtered through cotton wool (cotton wool was used for filtration due to the presence of the mycelium, which causes blockage of the

membranes filters). Antimicrobial activity was detected by the agar well diffusion method (Irshad *et al*., 2012). Single colonies of both *E. coli* ATCC 10536 and *S. aureus* ATCC 25923 were placed into Falcon tubes into 5 ml of normal saline and vortexed until the colonies were completely dispersed and adjusted to 0.5 McFarland turbidity standard with saline. Then the suspensions were spread on MHA plates with a cotton swab. Wells (10 mm diam.) were cut into the agar using a sterile cork borer and 1 ml of each filtrate was placed in the wells. The plates were incubated for at 37°C for 24 h and the diameters of clear zones around the wells measured.

2.8 Optimizing DNA Extraction from isolates

Both liquid and solid cultures were prepared before DNA extraction.

Liquid culture

A single colony of each antibiotic producing organism was inoculated aseptically into 30 ml of TSB and then incubated in a rotary shaker at 28 ^{o}C for 48 h at 180 rpm to allow the cultures to grow.

Solid culture

AIA plates were inoculated with single colony of each organism and the plates were incubated at 28^{o}C for 2 d for DNA extraction. Different DNA isolation kits were evaluated for extraction of the DNA from the isolates.

2.8.1 Master Pure (Gram Positive DNA Purification Kit) (Illumina, USA)

All the reagents, enzyme and tubes provided with the kit.

2.8.1.1 Sample Lysis

After a 48 h of incubation, approximately 1.0 ml of each culture was pelleted by centrifugation for 5 min at 10,000 x g (Fisher, Accuspin micro 17) and the supernatant discarded. Then 150 µl of TE Buffer was added to each pellet, vortexed to resuspend the pellets and 1 µl of Ready-Lyse Lysozyme (20-40 KU/µl) was added and incubated at 37 ^{o} C for 24 h. Proteinase K (1 µl of 50 µg/µl) was added to an Eppendorf tube containing 150 µl of Gram Positive Lysis Solution. This master mix (150 µl) was added to each samples and vortexed thoroughly. The samples were then incubated for 15 min at 65-70^{o}C and vortexed briefly every 5 min. The samples were then cooled to room temperature and placed on ice for 3-5 min.

2.8.1.2 DNA Precipitation

When the samples had cooled, 175 µl of MPC Protein Precipitation Reagent was added and vortexed vigorously for 10 sec and the tubes were centrifuged at 4^{o}C for 10 min at 10,000 x g (Labnet international, PrismR, USA). The pellet (unwanted proteins) was discarded and the supernatant was transferred into a new micro centrifuge tube. Then 1 µl of RNase A (5 µg/µl) was added to each sample, mixed thoroughly and incubated for 30 min at 37^{o}C. Then 500 µl of isopropanol (BDH Laboratory supplies, England) was added, mixed by inverting the tubes 30-40 times. The desired DNA was pelleted by centrifugation using the same conditions as before (4^{o}C for 10 min) and the isopropanol was removed carefully without dislodging the DNA. The pellets were then rinsed using 70% ethanol (100 µl) and left to completely dry out the ethanol at room temperature. Finally, the DNA was dissolved in 35 µl of SD pure water for further analysis.

2.8.2 *FastDNA* spin kit (MP Biomedical, France)

All the reagents, enzyme and tubs provided with the kit.

Cultures were incubated for 48 h in TSB medium and approx. 1.0 ml of each culture was pelleted by centrifugation and the supernatant discarded. From solid media a loopful of culture was taken. Pellets or the loopfuls of the culture were suspended in normal saline 0.9% (Fisher, UK) to give a maximum suspension volume of 200 µl, and the sample was added to Lysing Matrix A tube. Then 1 ml Cell Lysis Solution (CLS) was added to Lysing Matrix A tube, and a TissueLyser (TisseLyser II, Qiagen) was used to homogenize the samples (5 min at 27 Hz). Samples were then centrifuged at 14,000 x g for 5-10 min to pellet debris.

2.8.2.1 Binding DNA to matrix

The supernatant (700 – 800 µl) was transferred to a 2.0 ml microcentrifuge tube and an equal volume of Binding Matrix added. The tube was inverted to mix the contents then incubated for 5 min at room temperature on a rotator. Eight hundred microlitres of the suspension was transferred to a spin filter and centrifuged at 14,000 x g for 1 min, the catch tube was emptied and the remaining suspension added to the spin filter and centrifuged as before. The catch tube was emptied again and 500 µl prepared SEWS-M (wash solution) added. The pellet was gently resuspended using the force of the liquid from the pipette tip and the tube was centrifuged at 14,000 x g for 1 min.

The contents of the catch tube were discarded and the tube was replaced and centrifuged a second time at 14,000 x g for 2 min and the catch tube was replaced with a new, clean tube.

2.8.2.2 Elution and Collection of Final DNA

DNA was eluted by gently resuspending the Binding Matrix above the spin filter in 100 µl of DES incubated for 5 min at 55℃ in a heat block, which was then centrifuged at 14,000 x g for 1 min to force the eluted DNA into the clean catch tube. Finally the spin filter was discarded.

Six different DNA isolation kits including the Bioline Kit (Isolate II Genomic DNA Kit), the Gene All Cell (Tamar Laboratory Supplies Ltd.), the Isolate Fecal DNA Kit (MO BIO Laboratories) and the Powersoil DNA Isolation Kit (MO BIO Laboratories) were investigated for extraction of DNA from the isolates but the isolated DNA's were of low concentration and purity.

2.9 PCR amplification of the 16S rDNA and 18S rDNA gene

Extracted genomic DNA from each sample was then used as template for PCR targeting the 16S ribosomal DNA (rDNA) gene and 18S rDNA using primer sets shown in Table 2.3.

Table 2.3: Primers used to amplify the full 16S rDNA gene for identification of bacteria and 18S rDNA gene for identification of fungi

Name	Sequence	Ref
16S bacterial universal primers	F- 5' AGA GTT TGA TCC TGG CTC AG- 3'	(Weisburg *et al*., 1991)
	R- 5' ACG GCT ACC TTG TTA CGA CTT-3'	
18S fungal universal primers	F-5' GTA CAC ACC GCC CGTC-3'	(Amaral-Zettler *et al*., 2009)
	R-5'TGA TCC TTC TGC AGG TTC ACC TAC-3'	

The primers were obtained from Eurofins UK Ltd.

To amplify the 16S rDNA genes and 18S rDNA a standard Taq based PCR procedure was used. PCR reactions were set up using the reagents and volumes set out in Table 2.4 and the PCR conditions and cycles as in Table 2.5.

Table 2.4: The PCR reaction mixture for 16S rDNA gene and 18S rDNA amplification of cultured isolates

Component	**Amount**	**Concentration**
Taq DNA polymerase (Bioline, UK)	12 µL	(0.625 units/25 µl PCR)
Weisberg Forward Primer	1 µL	0.2 µM (0.05-1 µM)
Weisberg Reverse Primer	1 µL	0.2 µM (0.05-1 µM)
Template DNA	1 µL	Variable <100ng
Nuclease-free water (Sigma, UK)	10 µL	
Total volume	25 µL	

Table 2.5: 16S rDNA and 18S rDNA PCR cycling stages, temperatures and times

Stage	**Temperature**	**Time (MyTaq®)**	**Cycles**
Initial denaturation	95°C	1 min	1
Denaturation	95°C	30 sec	
Annealing	53°C	30 sec	25
Elongation	72°C	1 min	
Final	72°C	1 min	1

2.10 Gel Electrophoresis

After thermocycling, PCR products were immediately stored on ice. A 1% (w/v) agarose gel was prepared using 1 g of agarose in 100 ml 0.5% v/v TBE (Bioline, UK). The solution was then microwaved and boiled for 1 min until it was completely homogenised and placed on an orbital shaker to cool down for 20 min at room temperature. When the bottle was at approx. 50°C, 100 µl of Gel Red fluorescent

DNA stain (Biotium, USA) was added and the liquid swirled gently. The 100 mL mixture was then poured into a gel tray, the well forming comb was placed into the gel and the gel was left to set for 30-40 min. Samples were run at 100 v, 100 ma until the coloured markers (Red for the PCR product (5 µl) and blue for the 1 kb HyperLadder (Bioline, UK) 3 µl) were 3 cm from the end of the gel (~75 min). The gel was then viewed in a G:Box Chemi transilluminator (Syngene) to check for successful amplification and correct sizes of amplified genes.

2.11 Sequencing analysis

PCR products were sent to Source Bioscience Sequencing (Nottingham, UK) for purification and sequencing. The sequencing was performed using either of the primers used for PCR, forward and reverse reads were generated for all amplicons and analysed using FinchTV viewer (Version 1.4.0, Geospiza). Each chromatogram file of nucleotide sequences was individually checked for final use by using FinchTV viewer. Forward primer and reverse complement sequence were aligned by Omega Clustal (http://www.ebi.ac.uk/Tools/msa/clustalo). Consensus sequences were identified using BLAST function of GenBank database https://blast.ncbi.nlm.nih.gov/Blast.cgi.

A phylogenetic tree was generated by Omega Clustal after all sequences were aligned.

2.12 Production, Extraction of antibiotics from broth medium

2.12.1 Production of the antibiotics

Two loopfuls from 7 d cultures on AIA of the Es1 isolate were inoculated into sterile 50 ml portions of AIB in 250ml conical flasks and incubated at 28±2 ºC on a rotary shaker at 120 rpm for 3 d to obtain heavily inoculated suspensions. Fresh sterile AIB medium was aliquotted in 250 ml portions in conical flask of 1 L capacity, then plugged, sterilized, cooled and heavily inoculated with the suspension. The flasks were fixed to a rotary shaker at 28±2 ºC 120 rpm and shaken for 7 d. Cultures were then removed and filtered through cotton wool. Antibiotic activity was detected by the agar well diffusion method; 1 ml of each filtered medium was added to the wells made in seeded HMA plates inoculated with a lawn of test organisms as before.

2.12.2 Extraction of antibiotics

The clear filtrate of the medium was extracted with ethyl acetate by vigorous shaking for 1 h. The organic layer was then separated in a separating funnel (Fisher, UK), and evaporated to dryness by rotary evaporation at 45°C. The aqueous phase was tested by the agar well diffusion method but had no antibiotic activity and was discarded. The crude orange residue obtained was redissolved in ethyl acetate and antibacterial activity tested against *E.coli* ATCC10536 and *S. aureus* ATCC 25923 using the paper disc method (Bauer *et al*., 1966). Bacterial suspensions of two organisms were prepared to be tested against the extracted antibiotic from Es1. Two 6 mm filter paper diffusion discs (Whatman, UK) were each loaded with 20 µl of the extracted antibiotic and allowed to dry (the control was two paper discs loaded with ethyl acetate solvent). Lastly, using a sterilised dissection needle each disc was placed on a MHA plates that had been seeded with bacteria (*E.coli* ATCC 10536 and *S. aureus* ATCC 25923*)* as before, cooled in the fridge for 30 min to allow the discs to settle and incubated at 37^{o}C for 24 h and clear zone sizes recorded.
The mycelium was harvested after 7 d incubation by filtering the broth through cotton wool, extracted with 100 ml acetone by vigorous shaking for 1 h, then filtered through the cotton wool and assayed against the selected test organisms as above (the control was a paper disc loaded with acetone solvent as before).

2.12.3 Time optimization for enhancement of yield of antibiotics

Isolate Es1 was grown in 300 ml portions of AIB in conical flask of 1 L capacity at pH 7.0. The flasks were fixed to a rotary shaker at 28±2 ºC 120 rpm. Broth samples were taken 3, 5 and 12 d of incubation, filtered through cotton wool and tested for their antibacterial activities.

2.12.4 Production, Extraction of antibiotics from solid medium

2.12.4.1 Extraction of antibiotic

One isolate (Es1) that had been tested and had showed good antimicrobial activities was grown on two plates of AIA for 7 d 28±2 °C. Two different methods were used to extract the antibiotic, the first method used was homogenizing the agar with the ethyl acetate using a magnetic stirrer for 2 h, the second method involved use of a sonicator (Ultrasonic Bath Fischer FS140H, frequency of 40kHz), which was used
for 1 h.

A spatula was briefly flamed on a Bunsen flame. After this the spatula was used to cut into the agar of the sub cultured Es1 isolate into small pieces. The cut agar was then taken from the plate and placed into a clean flask or a beaker in a fume cupboard. Forty mililitres of ethyl acetate was added to the flask and then a small piece of tin foil was used to cover the flask. After being in the sonication machine or on the stirrer, the agar and ethyl acetate mixture was filtered through a sintered glass filter (Whatman, UK) and the filtered extracts were tested for antibacterial activity by the disc diffusion method. Two 6 mm filter paper diffusion discs were loaded with the solvent, 100 µl of the extracted antibiotic was loaded on each disc and allowed to dry for 20-30 min and the same process was repeated until the antibiotic concentration on each disc reached 300 µl, then tested as before.

2.12.4.2 Inoculation methods

Three different inoculation methods were tested to see which method would produce the highest antibiotic concentration. The inoculation methods were the zigzag, a straight streak and individual colonies, plated as shown in Figure 2.2.

Isolate Es1 was grown on AIA for 7 d 28±2 °C, using the three different inoculated methods (6 plates for each inoculation method), then the agar was cut into small pieces, put in flask, 120 ml ethyl acetate was added, sonicated for one hour then filtered. As before samples were loaded onto filter paper disks 6 mm (300 µl) then tested.

Figure 2.2: Es1isolate inoculated with different patterns. Individual colonies (left), Zigzag (middle) and Streak (right)

2.12.4.3 Scale up number of plates extracted

Subcultured Es1 isolate was inoculated on 20 AIA plates incubated at 28±2ºC for 7 d. The antibiotic activity was extracted with the sonicator method on two portions first one as above 6 plates in one beaker, second portion scaled up, 14 plates in one beaker with 280 ml of ethyl acetate, then the extracts were filtered, two 6 mm filter paper discs were loaded with each extract (300 µl) then tested as before.

2.12.4.4 Time of incubation

Es1 was subcultured on 12 AIA plates, incubated at 28±ºC, on day 7 of incubation 6 plates were removed from the incubator, the antibiotic was extracted by the sonicator method, the extracts were filtered, the filtered extract was labeled and stored at 4ºC. After 21 d the remaining 6 plates were extracted as before. Two 6 mm filter paper discs were loaded with each extract (300 µl) then tested as before.
Differences in culture timescales were used for production of antibiotics from broth and solid medium as preliminary studies showed this to be the best incubation periods to obtain the maximum yields of the antibiotics (Table 2.6)

Table 2.6: Summary of incubation times for enhancement of antibiotics yield from isolate Es1

Broth medium			Solid medium	
3 d	5 d	12 d	7 d	21 d

2.12.4.5 Different solvent for antibiotic extraction

Isolate Es1 was grown on AIA at 28 ºC for 21 d each. Four batches of 6 plates were cut up into small pieces, put in flask in a fume cupboard and 120 ml of ethyl acetate, 120 ml chloroform, 120 ml acetone and 120 ml combination of ethyl acetate + chloroform 1:1 were separately added to the 4 flasks to determine which solvent would give the highest extraction of the antibiotic from the solid medium. These were then sonicated for 1 h, filtered, loaded 300 µl of the filtrate on paper disk then tested as before.

2.13 Extraction of antibiotics from different isolates

Single colonies of each isolate were streaked over AIA plates, one colony per plate, using the zigzag method, and then incubated at 28±2ºC for 21 d. Six plates of each isolate were cut to small pieces, put in flask in a fume cupboard, 120 ml of ethyl acetate was added, sonicated for 1 h then filtered and 300 µl loaded onto paper disks then tested as before.

2.14 Large scale: Isolation of antibiotic produced by isolatesEs1, M1 11, and La1a

Three isolates that gave good antibiotic activity (Es1, M1 11 and La1a) were selected and 300 AIA plates were inoculated by the zigzag method for each isolate and incubated at 28±2ºC for 21 d. The antibiotics were then extracted by cutting up 6 plates put them in flask, 120 ml ethyl acetate was added to each flask, sonicated for 1 h, the solvent was, filtered then evaporated by rotary evaporator, the crude extract was dissolved in HPLC grade methanol.

All solution extracts were filtered again by 0.22 µm filters (Millex-GS Syringe Filter Unit, 0.22 µm, mixed cellulose esters, Sigma, UK) to prevent damage to the HPLC columns.

2.15 Quantitative determination of the antibacterial activity of the extracts

Dry weights of the extracts from isolates Es1, La1a and M1 11were determined by placing 2 ml crude extracts into weighed vials which were reweighed after evaporation of the solvent. Extracts were dissolved in ethyl acetate and adjusted to 1 mg ml^{-1}. Then 10 µl of each extract was loaded on paper disks, which were allowed to dry and placed the on plates seeded with *E.coli* ATCC 10536 and *S.aureus* ATCC 25923 which were then incubated at 37ºC for 24 h.

2.16 Calibration Graph

Different volumes (5 µl, 10 µl, 15 µl, 20 µl, and 25 µl) of the three extracts were loaded onto 6x6 mm filter paper disks and allowed to dry and antibiotic activity determined as before.

Two bacteria were used to create the calibration curve *(S. aureus* ATCC 25923 strain and *E. coli* ATCC 10536*).*

2.17: Purification and determination of the structure of the antibiotics

2.17.1 High Performance Liquid Chromatography: Purification of the antibiotics for Es1 extract

In this study, reverse phase, analytical and semi-preparative, high performance liquid chromatography (HPLC Agilent Technologies 1220 Infinity LC) was used for fractionation and purification of the extracts. Reverse-phase (RP) was chosen as this method works best for low molecular mass molecules as well as neutral compounds (Cseke *et al*., 2016). The success of HPLC separations in this study depends on the selection of mobile phases (solvent) and column type. Isocratic elution was used in this study to choose the best column and solvent system to use for fractionation and purification of the extracts, according to the study conducted by (Friesen *et al*., 2015), this method is easy to perform, reliable, and sufficient for separations, gradient elution was then used with semi-preparative column (Hichrom Ltd, UK) for collecting fractions with antimicrobial activity.

Solvent systems

HPLC (Agilent Technologies 1220 Infinity LC), analytical column, (HP Hewlett Packard Spherisorb ODS-2 5µm 250 mm x 4.6 mm I.D) was used. The separations were conducted by injecting 20 µl of the extract (from isolates Es1 and M1 11) onto the analytical column. Six solvent systems were used in this study for optimising the analytical RP-HPLC parameters. All systems used isocratic elution with different mobile phases (water: methanol 70:30 (v/v), water: methanol 50:50 (v/v), water: methanol 40:60 (v/v), water: methanol 30:70 (v/v), 30:70 (v/v) of water: methanol containing 0.1 % trifluoroacetic acid (TFA) and a ratio 10:90 (v/v) of water: methanol).

Chromatograms were obtained using a UV-VIS HPLC detector (Agilent 1200 series, G1311B), the solvent system that gave best separation was used and the peaks of the chromatogram collected separately (called fractions). One mililitre of each fraction was placed on to two 6 mm filter paper disks in a petri dish in a fume cupboard for 24 h to evaporate the solvent. The filter paper disks were placed on MHA plates seeded with *E.coli* ATCC 10536 and *S.aureus* ATCC 25923 and incubated at 37°C to identify the fractions containing compounds with antimicrobial properties.

Columns

Three other analytical columns, ACE Excel 5 C-18 amide, ACE Excel 5 SuperC18 and ACE Excel 5 CN-ES (Hichrom Ltd, UK) were tried, the system was an isocratic elution using a mobile phase of water: methanol 30:70 (v/v), the separations were conducted by injecting an equal amount of sample (20µl) onto the analytical columns. The peaks of the chromatogram of the column that gave the best separation were collected separately and again 1 ml of each fractions was assayed for antimicrobial activity as before.

Comparison of fractionation from Es1 extracts from solid medium (AIA), broth medium (AIB) and mycelium extraction obtained from dense cultures of Es1 in AIB.

HPLC Agilent Technologies 1220 Infinity LC, analytical ACE Excel 5 C-18 amide column was used. The chromatogram was conducted by injecting an equal amount of the samples and the peaks of the chromatogram were collected separately and the fractions tested for antibacterial activity.

Table 2.7: Summary of amounts of extract loaded on 6mm filter paper disc for different samples

Extract	Amount
After extraction of antibiotics from filtered broth medium **2.12.2**	20µl
After two different methods were used to extract the antibiotic **2.12.4.1**	300µl
After three different inoculation methods used **2.12.4.2**	300µl
After scale up number of plates extracted **2.12.4.3**	300µl
After 7 and 21d of incubation **2.11.4.4**	300µl
After different solvents were used for antibiotic extraction **2.12.4.5**	300µl
After extraction of antibiotics from different isolates **2.13**	300µl
After collecting HPLC fractions **2.17.1**	1000µl

2.17.2 Collection of the fractions with antimicrobial properties

Based on the analytical HPLC separation results obtained from the first stage, a semi-preparative ACE Excel 5 C-18 amide column, Gradient HPLC system with a mobile phase of water: methanol 30:70 (v/v) and a flow rate of 5 ml/min was used. The separations were conducted by automated injecting 500 µl of the sample (Es1extract) each time onto the semi-preparative column, automated collector collected each fraction with antibacterial activity. All fractions were evaporated by rotary evaporator, after that the crud of each fraction eluted on Methanol-d4, than analyzed by NMR spectroscopy to check purity of the antibiotics.

The system used was an isocratic elution using a mobile phase of water: acetonitrile (Fisher, UK) 33:67 (v/v) for each fraction to remove impurities from the antibiotics. Two hundred and fifty microlitres of the semi pure antibiotic was injected into a semi-preparative ACE Excel 5 C-18 amide column; the peaks of the chromatogram were collected separately, and antibiotic activity determined as before. Fractions from each run were pooled and the solvent evaporated by rotary evaporator, the dried extracts were dissolved in methanol-d4.

2.17.3 High Performance Liquid Chromatography: Purification of the antibiotics from isolate M1 11 extracts

The same procedure as above was used for purification the antibiotics produced by M1 11 isolate, except that the solvent system was different and to get pure antibiotic three different solvent system were used, Gradient HPLC system, mobile phase of water: acetonitrile 33:67(v/v) with a flow rate of 5 ml/min for collecting the fractions with antimicrobial properties, than an isocratic system was used with a mobile phase of water: methanol: acetonitrile 30:35:35 (v/v) to remove impurities from the antibiotics. When analyzed with NMR the proton spectrum showed that the antibiotics were not yet pure and a further purification step using an isocratic system with a mobile phase of water: methanol 30:70 (v/v) was used to further purify the antibiotics.

2.17.4 Identification of Chemical Structures

NMR spectroscopy

After purified fractions were obtained by HPLC all the samples were analyzed by NMR spectroscopy, all NMR measurements were performed on a Bruker Ultra-shield Avance AMX 400 MHz spectrometer, using 5-mm NMR tubes (Wilmad NMR tubes, 5 mm diam., precision 500 MHz Sigma-Aldrich, UK). Samples (5 mg of the compound for some fractions and the available amount of the compounds for rest of the fractions) were dissolved in a 600 µl deuterated methanol-D_4 (CD_3OD) or deuterated Dimethyl sulfoxide-D_6 (DMSO-D_6).

FTIR

FTIR analysis of the each compound was carried out with a Thermo Nicolet iS10 IR spectrometer following the manufacturer's instructions. For each sample, 16 scans were collected.

Mass Spectroscopy (MS) of the active compounds

MS for pure compounds (Es1 fractions) were obtained first by LC-MS (Agilent Technologies 6120 Quadrupole LC/MS) at the University of Salford by injecting 10µl of each compound into instrument in positive and negative ion mode. Then, accurate MS were obtained by sending samples to the Cambridge University Chemical Laboratory to confirm the final structures of the compounds.

2.18 Antimicrobial and anticancer activities

2.18.1 Minimum Inhibitory Concentration (MIC)

In this study, the minimal inhibitory concentration (MIC) of Es1, M1 11 and La1a whole extracts and Es1 and M1 11 purified fractions were determined by broth micro-dilution method (Andrews, 2001). Two test organisms *E.coli* ATCC 10536 and *S. aureus* ATCC 25923 were used; the compounds were dissolved in Dimethyl sulfoxide (DMSO) as solvent.

The UV spectrophotometer was balanced and MHB was used as blank to zero the absorbance to achieve desired suspension (0.1). Overnight cultures of *E. coli and S.*

aureus were measured at 600 nm; *E. coli* ATCC 10536 was 1.422 and *S. aureus* ATCC 25923 was 0.932.

The following calculation was done to find out the concentrations of the bacteria and the broth needed to accomplish the MIC procedure:

C1 x V1 = C2 x V2

E. coli ATCC 10536: 1.422 x X = 0.01 x 20 ml

X = 0.2 / 1.422

X = 0.14 ml x 1000

X = 140 µl of *E. coli* ATCC 10536 and 20 – 0.14 = 19.85 ml of broth

S. aureus ATCC 25923: 0.932 x X = 0.01 x 20 ml

X = 0.2 / 0.932

X = 0.21 ml x 1000

X = 210 µl of *S. aureus* ATCC 25923 and 20 – 0.21 = 19.78 ml of broth

The broth was then added into 2 Falcon tubes and the bacteria concentrations added separately to these tubes.

Compound plate

Fifty microliters of DMSO was added into each well in a sterilize 96-well microtitre plate except the first well of each row. One hundred microliters of each compound/whole extract were add to the first well in each row of the plate, then serial dilutions prepared by transferring 50 µl to the next well and mixing for each compound (the last well of each row was just DSMO as solvent control).

Dosing plate

One hundred microliters of MH Broth was add into each well in a sterilize 96-well plate then 10 µl of each compound from compound plat added and mixed.

Bacteria plate

At the final in a sterilize 96-well plate, 100 µl from dosing plate was loaded with 100 µl bacterial diluted suspension (the final volume of each well was 200µl). Gentamycin and MHB medium were added in two separate rows as positive control and negative control respectively.

The bacteria well plates were then incubated at 37^{o}C for 24 h, then the MIC results obtained using a Multiskan FC plate reader (Thermo Scientific) at a wavelength of 570 nm.

Each determination was determined in triplicate and the mean value recorded.

2.18.2 Anticancer activity

The compounds were screened by the anticancer research section and the method used to test the compounds is described below.

2.17.2.1 Cell lines

Two different cancer cell lines MDA-MB 468 (mammary gland/breast; derived from metastatic site: pleural effusion) Wang *et al*., (1997), MCF 7 (breast adenocarcinoma) Lee *et al*., (2015) were used to test for potential anticancer activity and HACAT cells lines (Human immortalized keratinocytes) Boukamp *et al*., (1988) were used as normal cells.

2.17.2.2 Maintenance of cells

Cell lines were growing by adhering to a plastic wall of T75 cell culture flasks (Thermo Fisher Scientific, UK).

Dulbecco's modified Eagle's medium (DMEM; Sigma, UK) was used as a growth medium and the temperature was maintained at 37°C and 5% CO_2. Sterility of the working environment was ensured by using Virkon and 70 % ethanol in cleaning all utensils used. Cultures were examined using an inverted microscope to observe the confluency and confirm the absence of contaminants (bacterial and fungal). DMEM media were replaced every 3 d.

2.17.2.3 Splitting Matured Confluent Cells

Cell cultures were divided once 75 % confluence was reached. The cells were washed with pre-warmed (37°C) sterile phosphate-buffered saline (PBS; Sigma, UK) using a volume equivalent to half of the volume of cell culture medium. This step was repeated 3-4 times to remove the remaining media. Three mililitres of trypsin-EDTA solution at 0.25% (w/v) was added into the culture flask. Flasks were rotated to ensure that the walls were covered with trypsin, this was incubated for 2-5 minutes at 37 °C to ensure that cells were detached from the wall of the flask. The cells were then resuspended in 5 ml pre-warmed medium (37 °C) to dilute and inactivate the trypsin. Detached cells were split into 2 new culture flasks containing pre-warmed medium, and the flasks incubated in an incubator (37℃ with 5% CO_2 environment)

2.17.2.4 Cell viability assay using MTT

MTT (3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyl tetrazolium bromide; (Sigma, UK) viability assay was conducted as described by Holst and Oredsso (2005). One hundred microlitres of cell suspension were seeded in 96 well microtitre plates to give 20,000 cells/ well. Twenty-four hours after seeding, a concentration of compounds ranging from 3.13 to 100 μg/mL were diluted in appropriate DMSO was added to the wells. DMSO was used as the negative control, the treated cells were then left for 24 h. Five mg/mL MTT reagent (Sigma, UK) was dissolved in PBS and added unto the 96 well plates. Further incubation was done for 4 h. MTT was removed from the medium and cultures which had formed purple colour from the formazan in MTT were dissolved in DMSO (200 μl). A Thermo Labsystem Multiskan Ascent plate reader using Ascent software - (Labsystems Oy, Helsinki, Finland) was used to measure absorbance at 540 nm and the inhibitory concentration (IC_{50}) calculated.

Chapter 3

Isolation and identification of antibiotic producing microorganisms from terrestrial invertebrates

3.1 Introduction

No single method has been discovered as the correct approach to selectively isolate organisms from environmental samples. Rather, various approaches of individual or collection of organisms are used to selectively isolate multiple types of fungi and actinomycetes from environmental samples. For example fungi have been enriched by plating serial dilutions of environmental samples onto potato dextrose media that contains compounds that inhibit unwanted bacterial growth but allows the growth of the target fungi. For example antibacterial agents can be added to the isolation media to prevent growth of bacteria that may overgrow fungal species (Awad *et al*., 2009). Recovery can be improved by enriching the environmental substrate before selective isolation or through chemical or physical treatment techniques (Ikechi–Nwogu and Elenwo, 2012)

Many "non-selective" media have been recommended for the isolation of streptomycetes. In these media, carbon sources include glucose, glycerol, mannitol, or starch as the carbon source and arginine or asparagine as the nitrogen source. A source of both nitrogen and carbon is chitin, which is frequently used. Such "non-selective" media are now known to favor the isolation of a narrow range of streptomycetes and\or fungi and do not support the growth of organisms with more exacting growth requirements (Sembiring and Goodfellow, 2008).

Chemical screening

Currently, the screening techniques available to detect antimicrobial activity of natural products are categorized into three groups: bio-autographic; diffusion and dilution techniques. The bio-autographic and diffusion approaches provide ideas of the presence or absence of substances with antimicrobial activity thus referred to as qualitative methods. However, through dilution techniques it is possible to determine minimal inhibitory concentration thus referred to as quantitative assays. Antimicrobial activities mentioned in the literature have been assessed using diverse collection of

methodologies, sensitivity degrees, amount of test compounds and microbial stains and are usually challenging to compare (Valgas *et al*., 2007).

Three major factors need to be considered when screening for new bioactive microbial compounds: selection of producing organisms, culture methods and detection. The singularity of the microorganism and the novelty of both specific activity and chemical structure will increase the probability of success in the first stage (Sajid *et al*., 2009). In order to elucidate outstanding natural molecular diversity for drug development, various methods that have been developed are target-directed biological, physicochemical and chemical screening strategies. To visualize the secondary metabolite pattern, a chemical screening using Thin-layer chromatography (TLC) with various staining reagents and HPLC-MS/MS enables the visualization of an almost complete picture of the secondary metabolites, also known as their metabolic fingerprint (Taddei *et al.*, 2006b).

Maximization of antibiotic yield and manipulation of carbon and nitrogen sources.

The manipulation of the type and concentration of nutrients formulating the culture medium can greatly affect antimicrobial production. The effect of carbon and nitrogen source has been the focal point of study for industries as well as research groups. Nitrogen is crucial in synthesizing enzymes that play a part in primary and secondary metabolism and may be part of the antimicrobial molecule (Merrick and Edwards, 1995). Thus depending on the biosynthetic pathways involved, nitrogen may affect antibiotic formation. Tawiah *et al*., (2012) and Charyulu and Gnanamani, (2010) noted that the type of nitrogen source, whether organic or inorganic, was involved in the synthesis of secondary metabolites. They showed that using an organic nitrogen source *Pseudomonas aeruginosa* gave a higher yield of uncharacterized antimicrobial metabolites compared to inorganic sources. Specifically they showed that asparagine was better used for antibiotic production by *P. aeruginosa* rather than inorganic nitrogen sources such as sodium and potassium nitrates and ammonium salts. Production of penitrem B by *P. aurantiogriseum* also differed with different nitrogen sources with KNO_3 giving greatest yield, followed by L-asparagine, $NaNO_3$, glycine, DL-aspartic acid and L-tryptophan (Surekha and Reddy, 1992). *Aspergillus flavus* and *Penicillium citrinum* expressed higher levels of antibacterial activity with yeast

extract when compared to other nitrogen sources such as NH_4Cl, $(NH_4)_2SO_4$ and $NaNO_3$ (Compaore *et al*., 2016a*)*. In a study on the growth of *Streptomyces hygroscopicus* and biosynthesis of rapamycin using different nitrogen sources carried out by Lee *et al*., (1997), six non-amino acid nitrogen compounds were examined as nitrogen source. $(NH_4)_2SO_4$ was the best with respect to formation of rapamycin, and supported cell growth comparable to the organic nitrogen sources used.

In study carried out by Khopade *et al*., (2012) the production and properties of a biosurfactant isolated from a marine *Streptomyces* species B3 was reported as being best with yeast extract as a nitrogen source.

In study carried on the effects of nitrogen compounds on production of L-asparaginase by *Streptomyces ginsengisoli* by using different nitrogen sources, beef extract, yeast extract, and peptone were added at a concentration of 0.2% (w/v) to Asparagine Dextrose Salt Broth keeping other ingredients constant, Peptone gave maximum enzyme production by this strain (Deshpande *et al.*, 2014). Soyabean was the best nitrogen source for Melanin production from a marine *Streptomyces* (Vasanthabharathi *et al*., 2013).

Carbon sources are also important in optimizing yields of secondary metabolites and slowly utilised carbon sources such as lactose or glycerol have been shown to be optimal for secondary metabolite production than rapidly utilisable glucose (Jia *et al*., 2009). *P. aurantiogriseum* responded differently to different carbon sources with D-xylose found to be the best carbon sources for penitrem B production (Surekha and Reddy, 1992). The best carbon sources for production of antibacterial compounds appeared to be starch and glucose for *Aspergillus flavus* and starch and sucrose for *Penicillium citrinum* (Compaore *et al*., 2016a*).*

Glucose is an excellent carbon source for growth of most actinomycetes, compared to glycerol. However, exceptions include for example *Streptomyces clavuligerus*, a cephamycin producer, which cannot utilize glucose, but glycerol favours its growth, while antibiotogenesis is inhibited (Khaliq *et al.*, 2009). The regulation of carbon utilisation is of central importance in the gene expression pathways for both morphological development and antibiotic production in *Streptomyces* spp. as reported by Watve *et al*., (2001). In a study carried out by (Mehdi *et al*., 2006) the *Streptomyces* spp. TN97 strain showed that the highest biological activities were also

obtained when glycerol or fructose at 1% (w/v) was added in tryptic soy broth (TSB) medium after 72 h of incubation at 250 rpm.

The aims of this part of the study were to isolate microorganisms from invertebrates to screen them for antibiotic activity and identify them to at least species level and to optimise antibiotic yields by culture selected organisms on different culture media.

3.2 Results

3.2.1 Isolation of antibiotic producing microorganisms

Twenty-one aquatic and 14 terrestrial invertebrates were investigated for the presence of *Streptomyces*-like organisms as described in section 2.1. A total of 125 microbial isolates were obtained and screened for antibiotic production.

3.2.2 Primary screening

3.2.2.1 Agar overlay technique

Organisms isolated from the invertebrates were initially screened by streaking on AIA agar and screened for antimicrobial activity against Gram positive (*S. aureus*) and Gram negative bacteria (*E.coli*) using the agar overlay technique.

3.2.2.2 Streak or single colony pattern on agar plates to detect antimicrobial activity

All of the isolates were tested for their activity against five test microorganisms. Typical results are shown in Figures 3.1 and 3.2 and the results are summarised in Table 3.1. The test microorganisms included three Gram positive bacteria (*S. aureus, S. aureus 2* and *B. cereus*) and two Gram negative bacteria *(E. coli* and *P. fluorescens*). The results showed that 47/125 (38%) isolates had promising antibacterial activity against one or more of the test organisms.

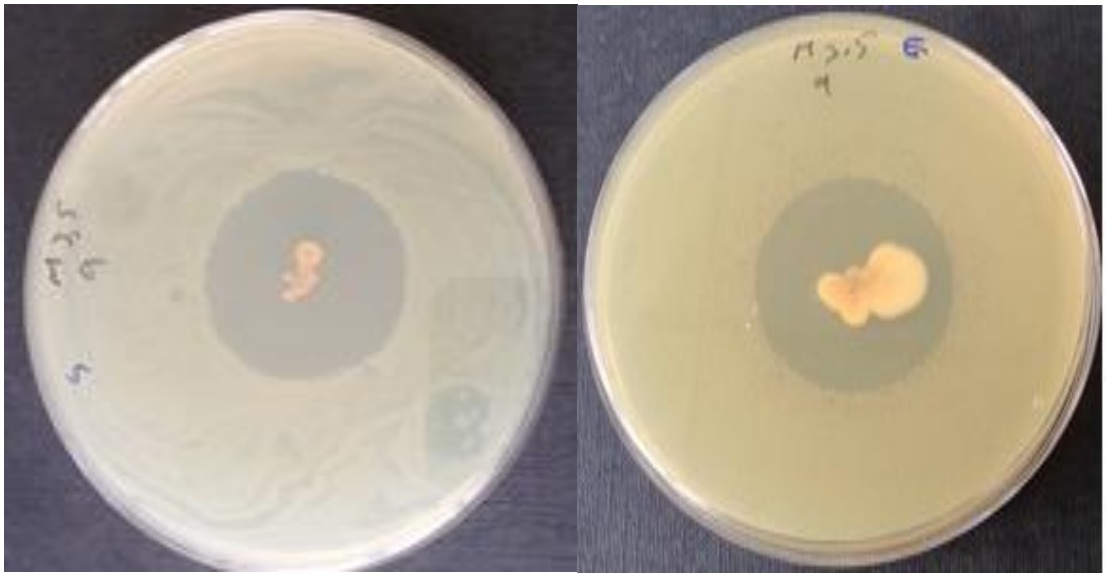
Table 3.1: Antibacterial activity against Gram positive and Gram negative bacteria

Invertebrates	**Isolates**	***E.c***	***S. a***	***S. a2***	***B. c***	***P. f***
Black worm	**La1a**	**++**	**++**	**++**	**++**	**+**
	La2	+	+	++	+	-
	La4	++	+	++	+	-
	La5	+	+	++	++	-
Whirligig Beetle Larva	M35 1	++	+	++	+	+
	M35 4	++	+	++	+	-
Sludge worm	M35 5	+	+	++	+	+
	M35 9	++	+	++	+	+
Biting midge larvae	M35 11	+	+	++	+	-
Swimming mayfly nymph	M1 2	-	+	++	+	-
	M1 3	+	+	+	+	-
	M1 4	-	+	++	++	-
	M1 8	-	+	++	++	-
	M1 10	-	+	++	++	-
	M1 11	**++**	**++**	**++**	**++**	**++**
Flattened mayfly nymph	M1 13	-	++	++	++	-
	M1 14	+	++	++	++	-
	M1 15	-	+	++	++	-
	M1 16	-	+	++	++	-
	M1 18	-	+	++	++	-
	M1 20	-	+	++	++	-
	M1 22	+	+	++	+	-
Red ant	Antw1	+	-	-	-	-
	Antw2	+	-	-	-	-
	Antw3	+	-	-	-	-
Yellow meadow ant	Antb4	+	-	-	-	-
Sea anemone	Sa1	-	-	+	-	-
	Sa2	-	-	+	-	-
Common wasps	W 1	-	-	+	+	-
	W 2	+	+	++	++	-
	W 3	-	+	+	+	-
	W 6	-	+	++	+	-
	W 7	+	+	++	+	+
	W 9	+	++	++	+	-
	W 12	+	+	++	+	-
	W 13	+	+	++	+	-

Invertebrates	**Isolates**	***E.c***	***S. a***	***S. a2***	***B. c***	***P. f***
Great Ram's-horn snail	Wata1	-	+	+	-	-
	Wata2	-	+	+	-	-
Ram's horn snail	p.p 1	-	+	+	-	-
	p.p 2	-	+	+	-	-
	p.p 3	+	+	+	-	-
Black slug	S 5	-	+	-	-	-
	S 6	-	+	-	-	-
Garden snail	**Es 1**	**++**	**++**	**++**	**++**	**++**
	Es 2	+	++	++	+	+
	Es 5	-	+	+	-	-
	Es 9	-	+	++	+	-

- No inhibition zone, + inhibition zone less 5mm, ++ inhibition zone more than 5 mm

E. c = E. coli ATCC10536, *S. a = S. aureus MOH1*, *S. a* 2 = *S. aureus* ATCC 25923, *B. c = B. cereus* ATCC 10876, *P. f = P. fluorescens* ATCC 13525.



a b

Figure 3.1: Agar overlay technique showing Inhibitory zone activity of the strain isolated from *Tubifex tubifex* (M35 9) against *S. aureus* ATCC 25923 *(a)* and *E. coli* ATCC10536 *(b).*

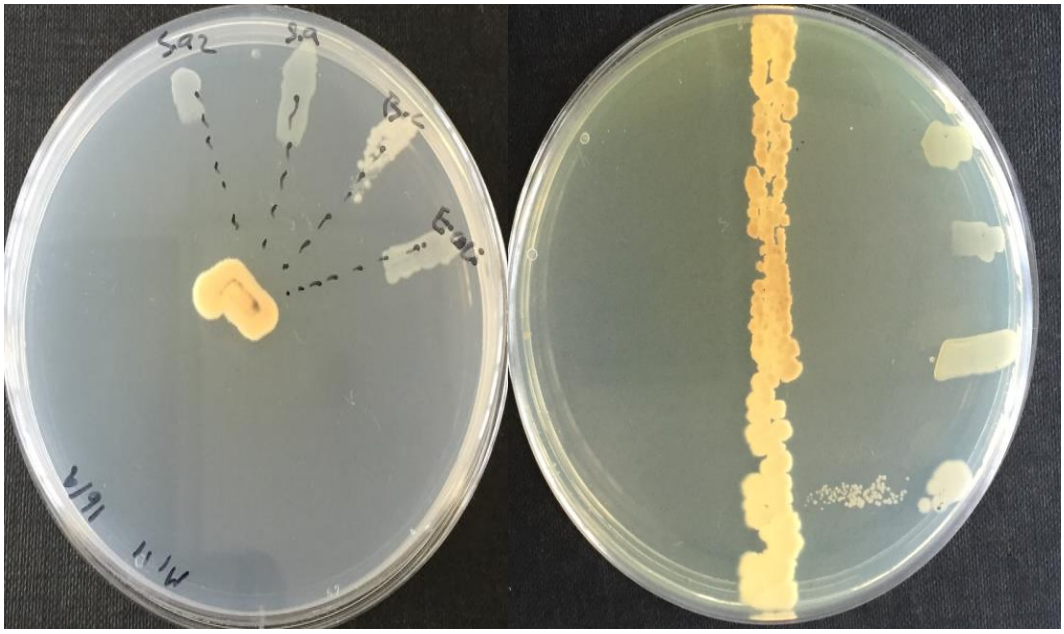


Figure 3.2: Streak and single colony pattern on agar plates showing inhibitory activity of the strain (M1 11) against test organisms.
The M1 11strain was isolated from Baetidae, *Swimming mayfly nymph* inhibited all 4 test organisms and showed inhibition of *S. aureus* AOH1 (Sa2), *S.aureus* ATCC 25923 (Sa), *E. coli* ATCC10536 (Ec) and *B. cereus* ATCC 10876 (BC).

Of the 47 isolates that showed antimicrobial activity, 28 were from aquatic invertebrates (Table 3.3) and 19 from terrestrial invertebrates (Table 3.4). Four strains were isolated from *Lumbriculus variegatus* (black worm), 2 from *Actinia equine* (sea anemone), 6 from *Baetis* spp. (swimming mayfly nymphs) 7 from *Baetis* spp. (flattened mayfly nymph), 2 from *Gyrinidae Beetle Larva* (whirligig), 2 from *Tubifex tubifex* (sludge worm), 1 from *Culicoides spp.* (biting midge larvae), 3 from *Planoris planoris* (ram's horn snail), 2 from *Planorbis corneus* (great Ram's-horn snail), 2 from *Arion ater* (black slug), 3 from *Myrmica rubra* (red ant), 1 from *Lasius Flavus* (yellow meadow ant), 4 from *Helix aspersa* (garden snail) and 8 from *Vespula vulgaris* (common wasp).

Table 3.2: Patterns of activity analysis

Activity	Isolates
Broad spectrum high activity	La1a1, M1 11, M1 14 and Es1
Broad spectrum weak activity	M35 5, M35 11, M1 3, M1 22, W7, W 12, W 13 and P.p3
Broad spectrum variable activity	La2, La4, La5, M35 1, M34 4, M35 9, W2, W9 and Es2
Gram positive only high activity	M1 4, M1 8, M1 10, M1 13, M1 15, M1 16, M1 18, and M1 20
Gram positive only low activity	Sa1, Sa2, W1, W3, Wata1, Wata 2, P.p1, P.p2, S 5, S 6, Es 5
Gram positive only variable activity	M1 2, W 6, Es 9
Gram negative only high activity	-
Gram negative only low activity	Antw1, Antw 2, Antw 3, Antb 4
Gram negative only variable activity	-

Broad spectrum high activity (inhibition zone more than 5 mm against at least three of five test organisms)

Broad spectrum weak activity (inhibition zone less 5 mm against at least four of five test organisms)

Broad spectrum variable activity (inhibition zone more than 5 mm against two test organisms but less 5 mm against the other three test organisms)

Gram positive only high activity (inhibition zone more than 5 mm against at least two of the three Gram positive test organisms)

Gram positive only low activity (inhibition zone less 5 mm against the three Gram positive test organisms)

Gram positive only variable activity (inhibition zone more than 5 mm against just one of the three Gram positive test organisms)

Gram negative only low activity (inhibition zone less 5 mm against the Gram negative test organisms)

Table 3.3: Organisms isolated from the aquatic invertebrates

Invertebrates	**Antibiotic producing isolates and strain designation**
Freshwater shrimp	0
Water flea	0
Bloodworm	0
A leech	0
Black worm	4 (la1a, la2, la4 and la5)
Bristle worm	0
Sea anemone	2 (Sa1 and Sa2)
Swimming mayfly nymph	6 (m1 2,3,4,8,10 and 11)
Flattened mayfly nymph	7 (m1 13,14,15,16,18,20 and 22)
Whirligig Beetle Larva	2 (m35 1, and 4)
Sludge worm	2 (m35 5 and 9)
Biting midge larvae	1 (m35 11)
Flatworm	0
Flatworm	0
Northern river crangonyctid	0
Waterlouse	0
Damselfly Nymphs	0
Ram's horn snail	3 (p. p1, 2 and 3)
Great pond Snail	0
Great Ram's-horn snail	2 (water s 1 and 2)
Waterlouse	0

Table 3.4: Organisms isolated from the terrestrial invertebrates

Invertebrates	**Antibiotic producing isolates and strain designation**
Roman snail	0
Yellow mealworm	0
Black slug	2 (S5 and S6)
Red ant	3 (Antw1, 2 and 3)
Yellow meadow ant	1 Antb4
22-spot Ladybird	0
Tiger worm	0
Common shiny woodlouse	0
Garden snail	4 (E.s1, 2,5 and 9)

Invertebrates	Antibiotic producing isolates and strain designation
Red flour beetle	0
Common wasps	8 (W1, 2, 3, 6, 7, 9, 12 and 13)
Yellow mealworm	0
Black ants	0
Common Earthworm	0
Red tiger worm	0

3.2.3 Secondary screening

Isolate Es1 from *Helix aspersa* showed the highest antimicrobial activity against a range of bacterial test species during primary screening. For this reason, the study focused largely on ES1 for secondary screening and production, extraction of antibiotics.

Comparative antibiotic production using different media

A loopful of isolate Es1 isolated from *Helix aspersa* was inoculated into 50 ml aliquots of the following media in 250 ml Ehrlenmeyer flasks: SB with $NaNO_3$ as source of nitrogen, SB with $(NH_4)_2SO_4$ as source of nitrogen, SB2 with $NaNO_3$ as source of nitrogen mixed with NB 2:1, SB2 with $(NH_4)_2SO_4$ as source of nitrogen mixed with NB 2:1, GNB, MEB, YEB, TSB and AIB. The inoculated media were incubated on a rotary shaker at 28±2ºC and 120 rpm for 7 d. One millilitre of filtered medium was placed in wells cut in MHA plates seeded with test organisms, incubated for 24 h and zone size determined. The results are shown in Table 3.5.

Filtered medium of SB with $NaNO_3$, SB with $(NH_4)_2SO_4$, MEB, YEB and AIB showed inhibition zones against *E. coli* ATCC10536 and *S. aureus* ATCC 25923. AIB, MEB and YEB promised to be the best media as they showed the largest inhibition zones.

Table 3.5: Antibiotic production for isolate Es1 using different media.

Test organism	SB +$NaNO_3$	SB +$(NH_4)_2SO_4$	SB2 +$NaNO_3$	SB2 +$(NH_4)_2SO_4$	GNB	MEB	YEB	TSB	AIB
S. aureus	4	3	-	-	-	14	12	-	14
E. coli	3	2	-	-	-	10	11	-	11

Inhibition zone diam. mm, - No inhibition zone

Key to media: SB +$NaNO_3$, Starch Broth with $NaNO_3$ as source of nitrogen; SB+$(NH_4)_2SO_4$, Starch Broth with $(NH_4)_2SO_4$ as source of nitrogen; SB2+$NaNO_3$ Starch Broth and Nutrient Broth 2:1 with $NaNO_3$ as source of nitrogen; SB2, Starch Broth and Nutrient Broth 2:1 with $(NH_4)_2SO_4$ as source of nitrogen; GNB, Glucose Nitrate Broth; TSB, Tryptone Soya Broth; MEB, Malt Extract Broth; YEB, Yeast Extract Broth; AIB, Actinomycete Isolation Broth.

3.2.4 Identification of isolates

3.2.4.1 DNA extraction

DNA was extracted from all isolates and tested for purity by using NanoDrop spectrophotometer. Six different DNA isolation kits (as described in section 2), were investigated for extraction of DNA from the isolates but the isolated DNA's were of low concentration and purity from five kits, Typical results were those obtained using the Bioline ISOLATE kits which were used for 2 isolates Es1 and M1 11 (from broth and solid media). The absorbance spectrum of the DNA samples is shown in Figure 3.3 and analysis details are shown in Table 3.6. The results show low purities and low concentrations.

#	Sample ID	User name	Date and Time	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type	Factor
1	Es1 Broth B	LABORATORY101	08/07/2016 15:47:42	1.1	ng/µl	0.022	0.013	1.66	0.59	DNA	50.00
2	Es1 Plate B	LABORATORY101	08/07/2016 15:50:11	1.0	ng/µl	0.019	0.017	1.13	0.46	DNA	50.00
3	M1 11 Broth B	LABORATORY101	08/07/2016 15:51:01	0.6	ng/µl	0.013	0.014	0.91	0.42	DNA	50.00
4	M1 11 Broth B	LABORATORY101	08/07/2016 15:51:28	0.6	ng/µl	0.013	0.010	1.20	0.55	DNA	50.00
5	M1 11 Plate B	LABORATORY101	08/07/2016 15:52:25	-0.1	ng/µl	-0.002	-0.002	0.76	-0.06	DNA	50.00
6	Es1 Plate M	LABORATORY101	08/07/2016 15:53:14	19.8	ng/µl	0.396	0.238	1.67	0.52	DNA	50.00
7	Es1 Broth M	LABORATORY101	08/07/2016 15:54:04	37.7	ng/µl	0.754	0.408	1.85	0.76	DNA	50.00

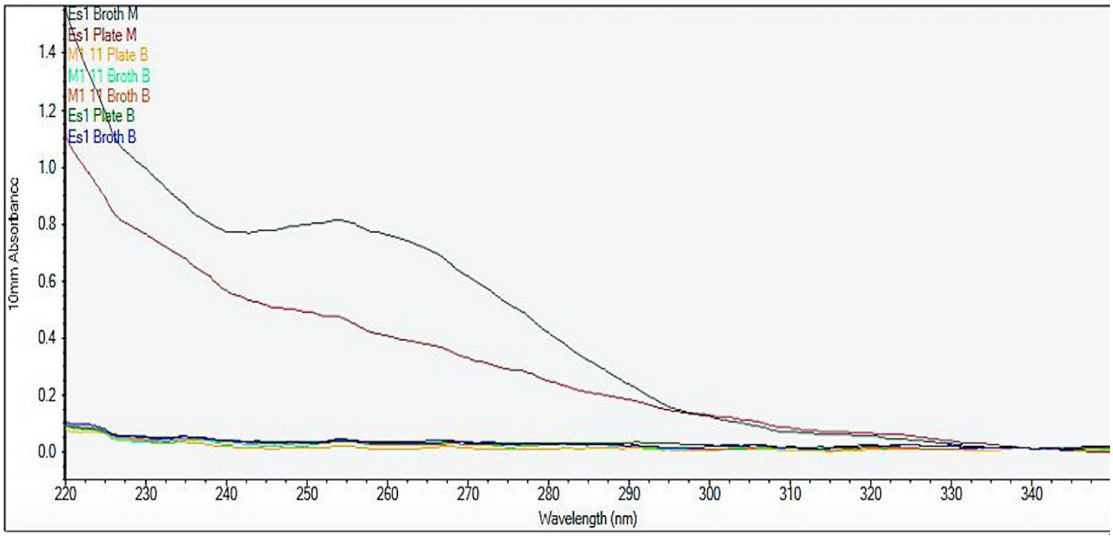


Table 3.6 and Figure 3.3: Raw spectroscopic data from NanoDrop spectrophotometer, showing absorbance at 260 and 280 nm for DNA extracted by Bioline kit.

However, when the FastDNA™ SPIN kit was used for DNA extraction for all isolates (from broth and solid media: Table 3.7 and Figure 3.4), the absorbance spectrum of the DNA samples showed acceptable purities and concentrations. Although not ideal, these extracts were found to be suitable for downstream applications such as PCR and sequencing.

#	Sample ID	User name	Date and Time	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type	Factor
1	pp1	LABORATORY101	18/08/2016 17:02:36	72.2	ng/µl	1.444	0.794	1.82	0.12	DNA	50.00
2	pp1	LABORATORY101	18/08/2016 17:04:26	40.8	ng/µl	0.816	0.466	1.75	0.05	DNA	50.00
3	pp3	LABORATORY101	18/08/2016 17:05:23	12.7	ng/µl	0.254	0.119	2.13	0.02	DNA	50.00
4	wata snail1	LABORATORY101	18/08/2016 17:06:26	38.8	ng/µl	0.777	0.409	1.90	0.05	DNA	50.00
5	wata snail2	LABORATORY101	18/08/2016 17:07:20	27.7	ng/µl	0.554	0.311	1.78	0.04	DNA	50.00
6	es2	LABORATORY101	18/08/2016 17:08:21	17.7	ng/µl	0.354	0.249	1.42	0.03	DNA	50.00
7	es5	LABORATORY101	18/08/2016 17:09:10	78.5	ng/µl	1.570	0.876	1.79	0.15	DNA	50.00
8	es9	LABORATORY101	18/08/2016 17:10:07	14.7	ng/µl	0.294	0.179	1.64	0.02	DNA	50.00
9	m1 11	LABORATORY101	18/08/2016 17:11:02	16.7	ng/µl	0.335	0.214	1.56	0.03	DNA	50.00
10	w2	LABORATORY101	18/08/2016 17:11:51	11.9	ng/µl	0.239	0.142	1.68	0.02	DNA	50.00
11	w7	LABORATORY101	18/08/2016 17:12:39	27.3	ng/µl	0.547	0.314	1.74	0.03	DNA	50.00
12	w7	LABORATORY101	18/08/2016 17:13:26	12.5	ng/µl	0.250	0.159	1.57	0.02	DNA	50.00
13	w12	LABORATORY101	18/08/2016 17:14:15	13.2	ng/µl	0.264	0.167	1.59	0.02	DNA	50.00
14	w13	LABORATORY101	18/08/2016 17:14:59	17.8	ng/µl	0.355	0.271	1.31	0.02	DNA	50.00
15	w18	LABORATORY101	18/08/2016 17:15:44	95.6	ng/µl	1.912	1.377	1.39	0.12	DNA	50.00

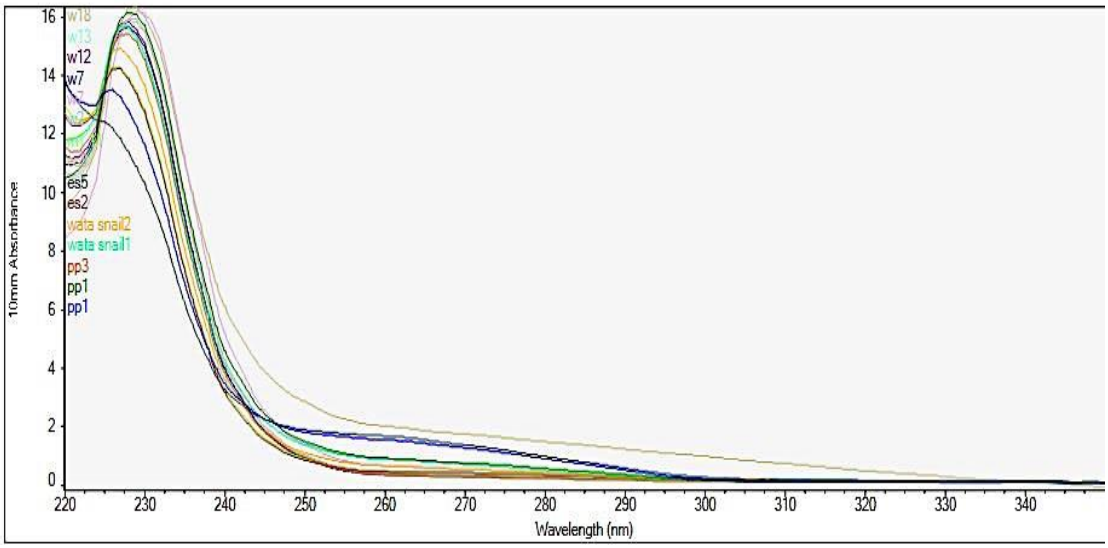


Table 3.7 and Figure 3.4: Raw spectroscopic data from NanoDrop spectrophotometer, showing absorbance at both 260 and 280 nm for DNA extracted using the FastDNA™ SPIN kit.

3.2.4.2 Polymerase Chain Reaction

PCR was performed using primers for 16S and 18S rDNA and examples of the agarose electrophoresis are shown in Figures 3.5 (18S primers) and 3.6 (16S primers). Bands of the expected size (240 for 18S and 1500 for 16S) were obtained.

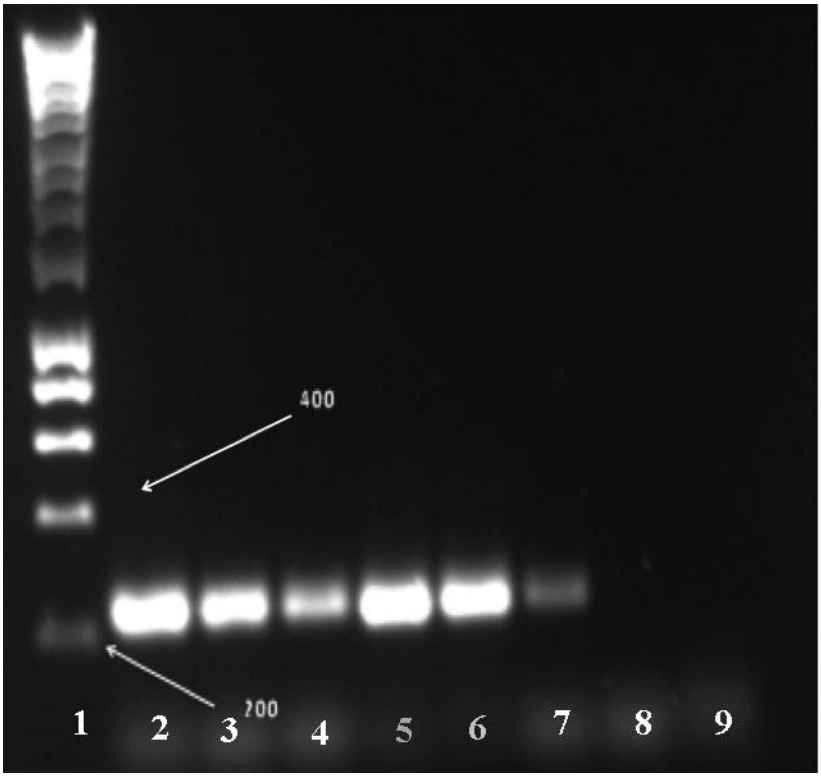


Figure 3.5: Amplification of 18S rDNA of *Penicillium* sp.; Lane 1: Hyperladder 1kb; Lanes 2-7 representing the samples (Es1, Es9, La1a, La2, M1 11 and M35 9 respectively); Lane 8-9: Negative control (no DNA).

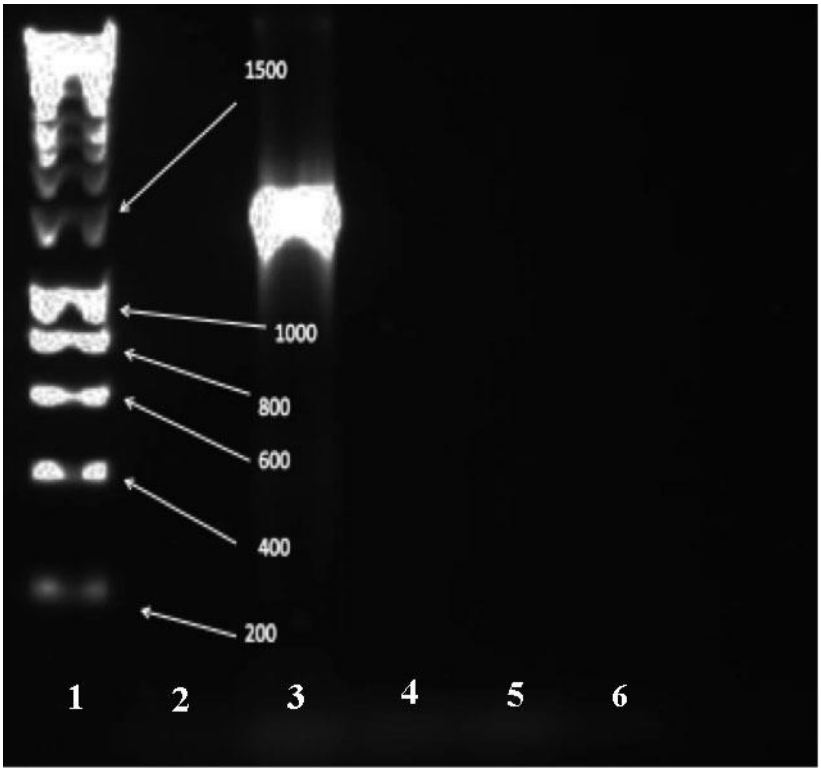


Figure 3.6: Amplification of 16S rDNA; Lane 1: Hyperladder 1kb; Lane 2 *Penicillium* sp (Es1); Lane 3 *Streptomyces* sp (Pp1); Lane 4 *Penicillium* sp (La1a); Lane 5 *Penicillium* sp (M1 11); Lane 6: Negative control (no DNA).

3.2.4.3 Sequence alignment and identification

The DNA from the 16S and 18S PCR experiments was sequenced and blast searched as described in section 2.11. The top 3 identification matches for each fungal isolate are shown in Table 3.8 and for bacterial isolates in Table 3.10.

Table 3.8: Sequence identification for fungal isolates

Isolate	ID	Similarity %	Accession	Reference
LA1A	*Penicillium brevicompactum* strain ATCC 16024	100	KP981369.1	(Sharifirad *et al*., 2016)
	Penicillium chrysogenum strain KCTC6052	100	AF411201.1	Published only in NCBI database 2001
	Uncultured eukaryote	100	LC109063.1	Published only in NCBI database
LA2	Uncultured *Penicillium* CHiv76 18S	100	KP974229.1	Published only in NCBI database 2015
	Uncultured fungus clone Aerocomp_EO40	100	FJ754892.1	(Le Goff *et al*., 2010)
	Penicillium commune strain IBT 15141	99	AF548089.1	(Wu *et al*., 2003)
LA4	*Penicillium tardum* strain KCTC16051	100	AF245233.1	Published only in NCBI database 2000
	Penicillium chrysogenum strain 215	100	GQ152991.1	(Davolos and Pietrangeli, 2007)
	Penicillium expansum	100	AB028137.1	Published only in NCBI database 1999
LA5	*Eupenicillium crustaceum*	100	D88324.1	(Ogawa *et al*., 1997)
	Penicillium chrysogenum strain 215	100	GQ152991.1	(Davolos and Pietrangeli, 2007)
	Penicillium chrysogenum strain KCTC6052	100	AF411201.1	Published only in NCBI database 2001

Table 3.8: Sequence identification for fungal isolates

Isolate	ID	Similarity %	Accession	Reference
M1 2	*Penicillium tardum* strain KCTC16051	100	AF245233.1	Published only in NCBI database 2000
	Penicillium brevicompactum strain ATCC 16024	100	KP981369.1	(Sharifirad *et al*., 2016)
	Eupenicillium crustaceum	100	D88324.1	(Ogawa *et al*., 1997)
M1 3	*Penicillium fimorum* strain DTO 159-F1	98	KU904343.1	(Houbraken *et al*., 2016)
	Penicillium fimorum strain CBS 140576	98	KU904342.1	(Houbraken *et al*., 2016)
	Penicillium sp DR1	98	KT805951.1	Published only in NCBI database 2015
M1 4	*Penicillium sp*. 2 BRO-2013	97	KF367494.1	(Oliveira et al., 2013)
	Uncultured fungus clone T3_II_1a_04	96	EF628511.1	(Poll *et al*., 2010)
	Penicillium sp HT-M15-LS	96	KJ527027.1	Published only in NCBI database 2014
M1 8	*Penicillium roqueforti* ATCC 10110	99	GQ458035.1	(Arteau *et al*., 2010)
	Penicillium camemberti strain ATCC 10387	99	GQ458039.1	(Arteau *et al*., 2010)
	Penicillium dipodomyicola strain ACBF 002-3	99	GQ161752.1	Published only in NCBI database 2009

Table 3.8: Sequence identification for fungal isolates

Isolate	ID	Similarity %	Accession	Reference
M1 10	*Penicillium tardum* strain KCTC16051	100	AF245233.1	Published only in NCBI database 2000
	Penicillium chrysogenum KCTC6052	100	AF411201.1	Published only in NCBI database 2001
	Penicillium expansum	100	AB028137.1	Published only in NCBI database 1999
M1 11	*Penicillium chrysogenum* strain 215	100	GQ152991.1	(Davolos and Pietrangeli, 2007)
	Eupenicillium crustaceum	100	D88324.1	(Ogawa *et al*., 1997)
	Penicillium brevicompactum strain ATCC 16024	100	KP981369.1	(Sharifirad *et al*., 2016)
M1 13	*Penicillium brevicompactum* ATCC 16024	100	KP981369.1	(Sharifirad *et al*., 2016)
	Penicillium chrysogenum strain KCTC6052	100	AF411201.1	Published only in NCBI database 2001
	Eupenicillium crustaceum	100	D88324.1	(Ogawa *et al*., 1997)
M1 14	*Penicillium expansum*	100	AB028137.1	Published only in NCBI database 1999
	Penicillium chrysogenum strain 215	100	GQ152991.1	(Davolos and Pietrangeli, 2007)
	Penicillium tardum KCTC16051	100	AF245233.1	Published only in NCBI database 2000
M1 15	*Penicillium solitum* strain 20-01	99	JN642222.1	(Eldarov *et al.*, 2012)
	Penicillium sp clone NJ-F4 18S	99	GU190185.1	Published only in NCBI database 2009
	Penicillium dipodomyicola strain ACBF 002-3	99	GQ161752.1	Published only in NCBI database 2009

Table 3.8: Sequence identification for fungal isolates

Isolate	ID	Similarity %	Accession	Reference
M1 16	*Penicillium roqueforti* strain ATCC 10110	100	GQ458035.1	(Arteau et al., 2010)
	Penicillium sp BF79	100	AM901692.1	(Pitkäranta *et al*., 2008)
	Penicillium sp BF18	100	AM901680.1	(Pitkäranta *et al*., 2008)
M1 18	*Penicillium sclerotigenum* strain NRRL 22813	100	DQ339574.1	(Dombrink-Kurtzman, 2007)
	*Penicilliumvulpinumstrain*NRRL1002	100	DQ339572.1	(Dombrink-Kurtzman, 2007)
	Penicillium vulpinum strain NRRL 2031	100	DQ339569.1	(Dombrink-Kurtzman, 2007)
M1 20	*Penicillium clavigerum* NRRL 1003	99	DQ339555.1	(Dombrink-Kurtzman, 2007)
	Penicillium griseofulvum NRRL 3523	99	DQ339549.1	(Dombrink-Kurtzman, 2007)
	Penicillium expansum strain NRRL 35259	99	DQ339548.1	(Dombrink-Kurtzman, 2007)
M1 22	*Penicillium brevicompactum* ATCC 16024	100	KP981369.1	(Sharifirad *et al*., 2016)
	Penicillium chrysogenum strain KCTC6052	100	AF411201.1	Published only in NCBI database 2001
	Penicillium expansum	100	AB028137.1	Published only in NCBI database 1999
ES1	*Penicillium brevicompactum* strain ATCC 16024	100	KP981369.1	(Sharifirad *et al*., 2016)
	Penicillium chrysogenum strain KCTC6052	100	AF411201.1	Published only in NCBI database 2001
	Eupenicillium crustaceum	100	D88324.1	(Ogawa *et al*., 1997)

Table 3.8: Sequence identification for fungal isolates

Isolate	ID	Similarity %	Accession	Reference
ES2	*Penicillium brevicompactum* strain ATCC 16024	96	KP981369.1	(Sharifirad *et al*., 2016)
	Penicillium chrysogenum strain 41H1	96	KX610138.1	Published only in NCBI database 2014
	Uncultured fungus clone CMH605	96	KF800692.1	(Rittenour *et al*., 2014)
ES9	*Penicillium chrysogenum* strain 215	100	GQ152991.1	(Davolos and Pietrangeli, 2007)
	Penicillium expansum	100	AB028137.1	Published only in NCBI database 2002
	Penicillium chrysogenum strain KCTC6052	100	AF411201.1	Published only in NCBI database 2001
ANT W1	*Verticillium fungicola* solate ARSEF 2065	100	AF108487.1	(Bidochka *et al*., 1999)
	Simplicillium lamellicola isolate UAMH 2055	100	AF108489.1	(Bidochka *et al*., 1999)
	Phytocordyceps ninchukispora strain CCRC 31900	100	AJ242434.1	Published only in NCBI database 1999
ANT W2	*Lecanicillium* sp strain CCF 5201	89	LT548278.1	Published only in NCBI database 2016
	Lecanicillium sp CCF 5233	89	LT548277.1	Published only in NCBI database 2016
	Fungal sp. SLZ14	89	KJ158154.1	Published only in NCBI database 2014
ANT W3	*Simplicillium lamellicola* isolate UAMH 2055	100	AF108489.1	(Bidochka *et al*., 1999)
	Cordyceps sp ATCC 36337	100	AJ242433.1	Published only in NCBI database 1999
	Fungal sp SLZ46	100	KJ183213.1	Published only in NCBI database 2014

Table 3.8: Sequence identification for fungal isolates

Isolate	ID	Similarity %	Accession	Reference
ANTB 4	Uncultured marine ascomycete clone PRTBE7359	100	HM800024.1	(Eloe *et al*., 2011)
	Uncultured marine ascomycete clone PRTBE7237	100	HM799960.1	(Eloe *et al*., 2011)
	Exophiala oligosperma strain CBS 725.88	99	AY554287.1	Published only in NCBI database 2004
M35 1	*Penicillium roqueforti* strain ATCC 10110	100	GQ458035.1	(Arteau *et al*., 2010)
	Penicillium dipodomyicola strain ACBF 002-3	100	GQ161752.1	Published only in NCBI database 2009
	Penicillium sp BF79	100	AM901692.1	(Pitkäranta *et al*., 2008)
M35 4	*Penicillium chrysogenum* strain 215	100	GQ152991.1	(Davolos and Pietrangeli, 2007)
	Penicillium expansum	100	AB028137.1	Published only in NCBI database 2002
	Penicillium tardum KCTC16051	100	AF245233.1	Published only in NCBI database 2000
M35 5	*Penicillium brevicompactum* strain ATCC 16024	100	KP981369.1	(Sharifirad *et al*., 2016)
	Penicillium chrysogenum KCTC6052	100	AF411201.1	Published only in NCBI database 2001
	Eupenicillium crustaceum	100	D88324.1	(Ogawa *et al*., 1997)

Table 3.8: Sequence identification for fungal isolates

Isolate	ID	Similarity %	Accession	Reference
M35 9	*Penicillium glandicola* NRRL 985	100	DQ339573.1	(Dombrink-Kurtzman, 2007)
	Penicillium vulpinum strain NRRL 1002	100	DQ339572.1	(Dombrink-Kurtzman, 2007)
	Penicillium glandicola strain NRRL 2036	100	DQ339565.1	(Dombrink-Kurtzman, 2007)
M35 11	*Penicillium* sp HT-M15-L	100	KJ527026.1	Published only in NCBI database 2014
	Uncultured fungus clone CMH605	100	KF800692.1	(Rittenour *et al*., 2014)
	Uncultured fungus clone CMH224	100	KF800315.1	(Rittenour *et al*., 2014)
Pp3	*Penicillium commune* strain MA09-AL	98	GQ458026.1	Published only in NCBI database 2009
	Penicillium coccotrypicola strain BRIP 59608	98	KM605436.1	Published only in NCBI database 2014
	Penicillium glandicola strain NRRL 985	98	DQ339573.1	(Dombrink-Kurtzman, 2007)
S 5	Uncultured *Penicillium* clone USN5	100	JN397370.1	Published only in NCBI database 2011
	Penicillium sp strain Y12 EG-2010	100	HM161749.1	(Galiana *et al*., 2011)
	Penicillium glabrum strain KCTC16099	100	AF245270.1	Published only in NCBI database 2000
S 6	*Fungal* sp Isolate nussu_195	100	KT714176.1	Published only in NCBI database 2013
	Fungal sp SLZ62	100	KJ183214.1	Published only in NCBI database 2014
	Hypocrea sp SP-4	100	HM152771.1	Published only in NCBI database 2010

Table 3.8: Sequence identification for fungal isolates

Isolate	ID	Similarity %	Accession	Reference
Sa 1	*Penicillium expansum*	100	AB028137.1	Ogawa., *et al* 2002
	Penicillium chrysogenum strain 215	100	GQ152991.1	(Davolos and Pietrangeli, 2007)
	Eupenicillium crustaceum	100	D88324.1	(Ogawa *et al.*, 1997)
Sa2	*Trichocomaceae sp.* LM196	100	EF060542.1	Published only in NCBI database 2006
	Uncultured *ascomycete*	100	AF290082.2	Lopez-Garcia., *et al* 2000
	Penicillium griseofulvum	100	DQ083547.1	Published only in NCBI database 2005
W1	*Penicillium freii* (IBT 3464)	99	AJ005446.1	(Skouboe *et al*., 2000)
	Penicillium dipodomyicola strain B64	99	KX090305.1	Published only in NCBI database 2016
	Penicillium gladioli strain NRRL 939	99	DQ339568.1	(Dombrink-Kurtzman, 2007)
W2	*Penicillium paneum* strain NRRL 25162	99	DQ339571.1	(Dombrink-Kurtzman, 2007)
	Peni*cillium solitum* strain 20-01	99	JN642222.1	(Eldarov *et al*., 2012)
	Penicillium echinulatum isolate 00044	99	DQ339568.1	(Dombrink-Kurtzman, 2007)
W3	*Penicillium coccotrypicola* strain BRIP 59608	99	KM605436.1	Published only in NCBI database 2014
	Penicillium tardum strain KCTC16051	99	AF245233.1	Published only in NCBI database 2000
	Penicillium griseofulvum	99	DQ083547.1	Published only in NCBI database 2005

Table 3.8: Sequence identification for fungi isolates

Isolate	ID	Similarity %	Accession	Reference
W6	*Penicillium brevicompactum* strain ATCC 16024	99	KP981369.1	(Sharifirad *et al.*, 2016)
	Penicillium chrysogenum strain KCTC6052	99	AF411201.1	Published only in NCBI database 2001
	Eupenicillium crustaceum	99	D88324.1	(Ogawa *et al*., 1997)
W7	*Penicillium vulpinum* NRRL 1002	99	DQ339572.1	(Dombrink-Kurtzman, 2007)
	Penicillium paneum NRRL 25162	99	DQ339571.1	(Dombrink-Kurtzman, 2007)
	Penicillium gladioli strain NRRL 939	99	DQ339568.1	(Dombrink-Kurtzman, 2007)
W9	*Penicillium* sp clone NJ-F4 18S	99	GU190185.1	Published only in NCBI database 2009
	Uncultured fungus clone CMH067	99	KF800158.1	(Rittenour *et al*., 2014)
	Uncultured fungus clone CMH605	99	KF800692.1	(Rittenour *et al*., 2014)
W12	*Penicillium expansum*	100	AB028137.1	Ogawa., *et al* 2002
	Penicillium chrysogenum strain 215	100	GQ152991.1	(Davolos and Pietrangeli, 2007)
	Eupenicillium crustaceum	100	D88324.1	(Ogawa *et al*., 1997)
W13	*Penicillium brevicompactum* strain ATCC 16024	100	KP981369.1	(Sharifirad *et al*., 2016)
	Penicillium chrysogenum strain KCTC6052	100	AF411201.1	Published only in NCBI database 2001
	Eupenicillium crustaceum	100	D88324.1	(Ogawa *et al*., 1997)

Thirty-seven isolates were confirmed as *Penicillium* spp. with similarities of 96-100% to previously published sequences. However, it was not possible to identify down to species level. The exceptions were Ant W1, 2 and 3, Ant B4 and S6. Examples of sequence alignments for La1a isolate with the three closest identification strains are shown in Figures 3.8-3.10. The similarity between the isolates was determined using Clustal Omega and the results are shown in Figure 3.7. Cluster analysis of fungal strains showed four main four clusters, fungal isolated from ants formed a separate cluster, Es1 and La1a clustered very close together and in the same branch with M1 11.

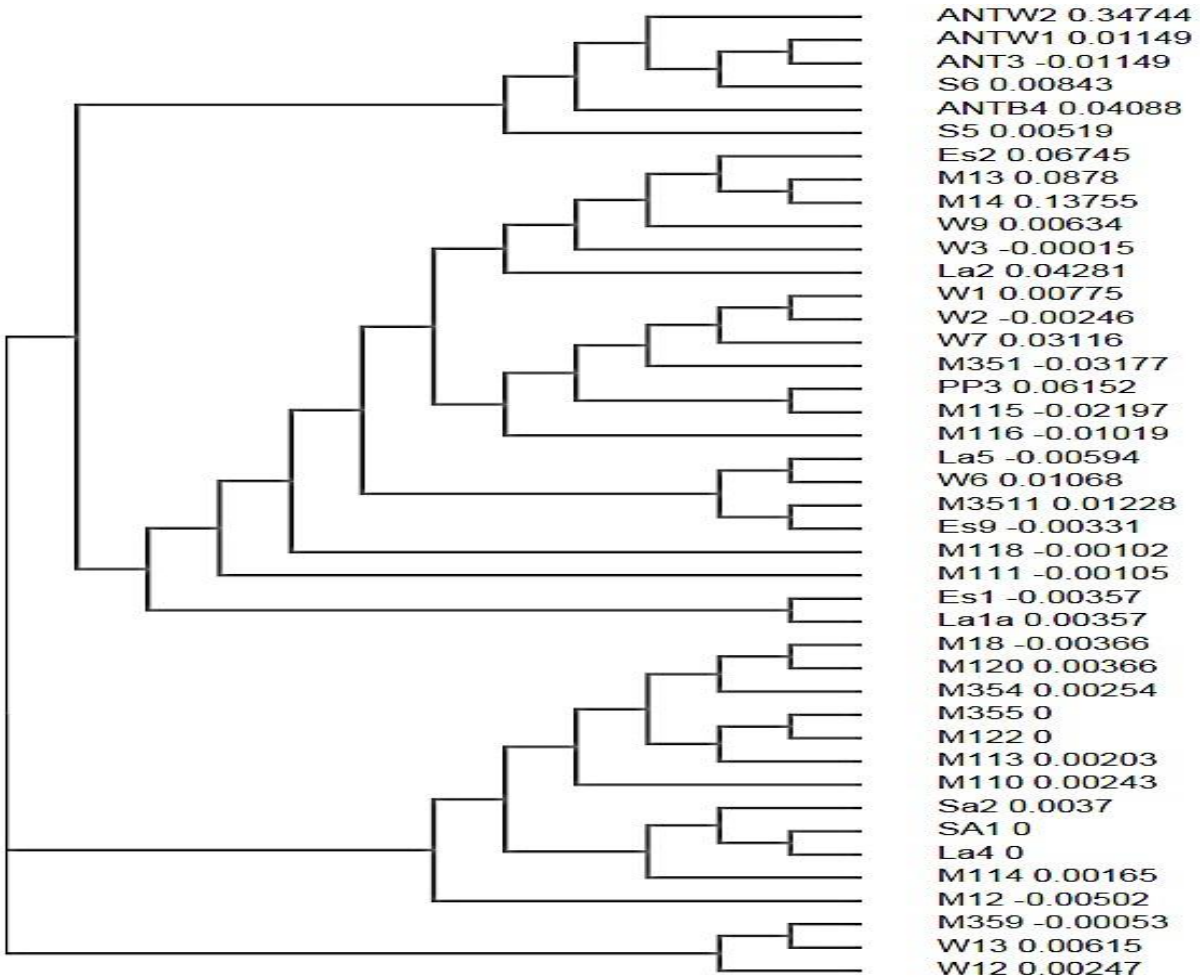


Figure 3.7: Phylogenetic relationships of 42 isolates indicating four main clusters, sequences were aligned using Clustal Omega and then the concatenated tree was generated.

Figure 3.8: *Penicillium brevicompactum* strain ATCC 16024

Figure 3.9: *Penicillium chrysogenum* strain KCTC6052

Figure 3.10: Uncultured eukaryote

ITS sequencing of the selected potent isolates for identification

Three strains were also sent for identification to an external source for confirmation and identification for species status.

Three plated culture chosen isolates (Es1, M1 11 and La1a) were sent to NCIMB LTD for ITS sequencing.

Identification Summary

Table 3.9: Result of ITS sequencing for potent isolates (Es1, M1 11 and La1a)

Isolation Code	Identification	% Similarity	Match
Es1	*Penicillium* species	100	Genus
La1a	*Penicillium* species	100	Genus
M1 11	*Penicillium chrysogenum*	99.8	Species

ITS sequencing was performed on the above isolates to distinguish between the suspected *Penicillium* group. Despite obtaining good sequences for all three isolates, only one isolate returned a species level match. An EMBL search was performed for all three isolates and the only published match to species level was to *Penicillium chrysogenum* for M1 11 (Appendix 1.43). There were *Penicillium chrysogenum* matches within the searches for La1a and Es1, but these were from unpublished sources. An alignment of all three isolates shows them to be very similar with no base differences (Appendix 1.45). Sample M1 11 was slightly longer, which may be the reason for the species level match.

Table 3.10: Sequence identification for bacterial isolates

Isolate	ID	Similarity %	Accession	Reference
Wata 1	*Streptomyces griseolus* strain 11-11	99	KJ571072.1	Published only in NCBI database 2014
	Streptomyces verne strain: NBRC 3112	99	AB184727.2	Published only in NCBI database 2004
	Streptomyces sp NBRC 3111	99	AB301481.1	Tagawa *et al.*, 2008
Wata 2	*Streptomyces* sp SIA-Cu-E1	99	KX056510.1	Published only in NCBI database 2016
	Streptomyces halstedii strain NRRL B-1238	99	NR_044148.1	Published only in NCBI database 2011
	Streptomyces cyaneus strain ISP 5108	99	NR_114820.1	Published only in NCBI database 2014
Es5	*Streptomyces* sp GS01	92	JX430438.1	Published only in NCBI database 2012
	Streptomyces fimicarius strain G8A-2	92	HQ238410.1	Published only in NCBI database 2010
	Streptomyces fimicarius strain G8A-17	92	HQ238384.1	Published only in NCBI database 2010
Pp1	*Streptomyces* sp GY2	95	AB907698.1	Published only in NCBI database 2014
	Streptomyces sp C3001	95	EU348370.1	Published only in NCBI database 2007
	Streptomyces parvulus strain K-15	94	KY038196.1	Published only in NCBI database 2016
Pp2	*Streptomyces sp.* strain 194	99	KX641400.1	(Betancur *et al*., 2016)
	Streptomyces sp. strain 184	99	KX641399.1	(Betancur *et al*., 2016)
	Streptomyces sp. strain CAE3	99	KX242156.1	Published only in NCBI database 2016

The results confirmed that all 5 strains were *Streptomyces* spp.. Strains Wata1, Wata 2 and PP2 were 99% similar to previously sequenced strains but it was not possible to confirm the species. Strain Es5 was possibly *S. fimicarius* but the match was only 92%.
Example sequence alignments for Wata 1 isolate with the three closest identification strains are shown in 3.11-3.13. All three sequences are identical and show a single mis-match at position 29.

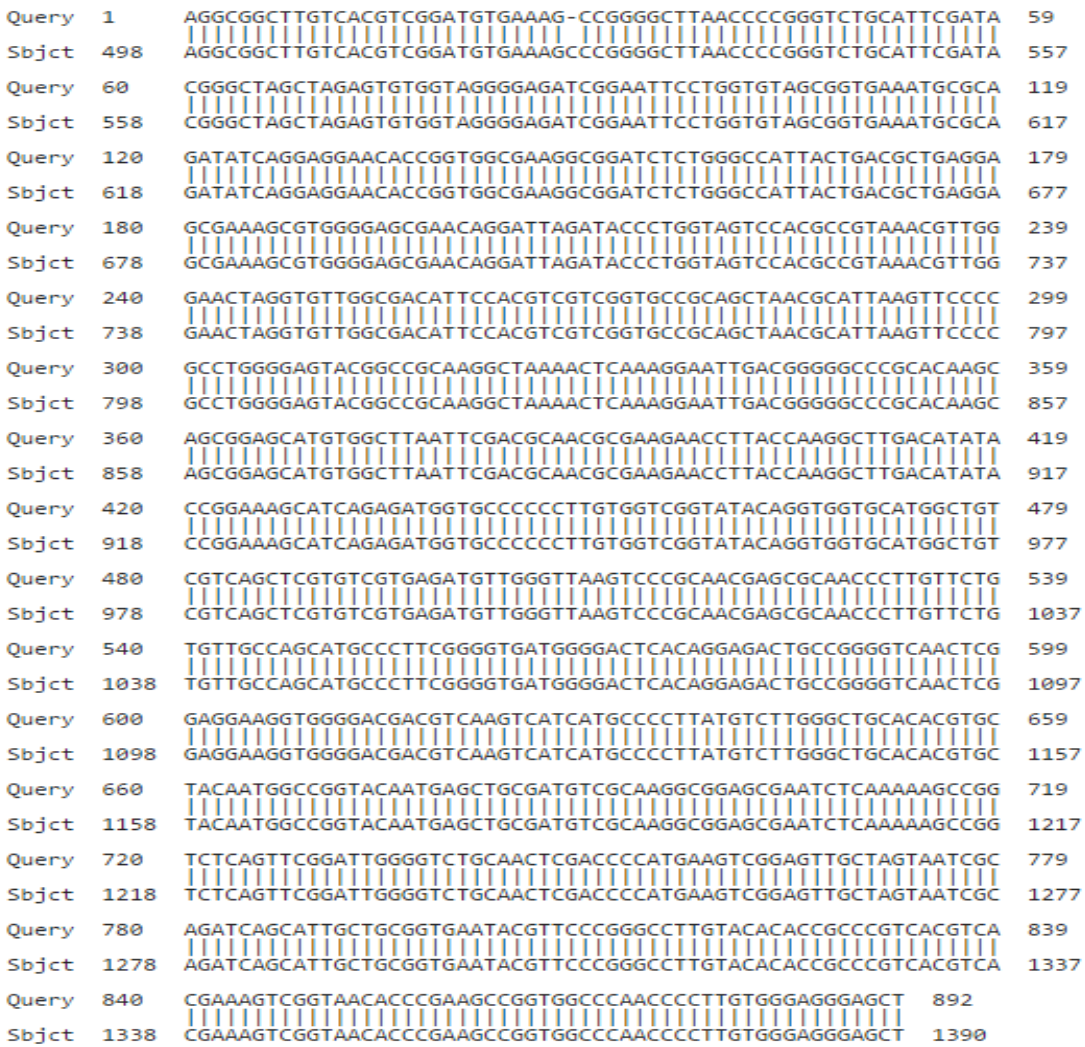


Figure 3.11: *Streptomyces griseolus* strain 11-11

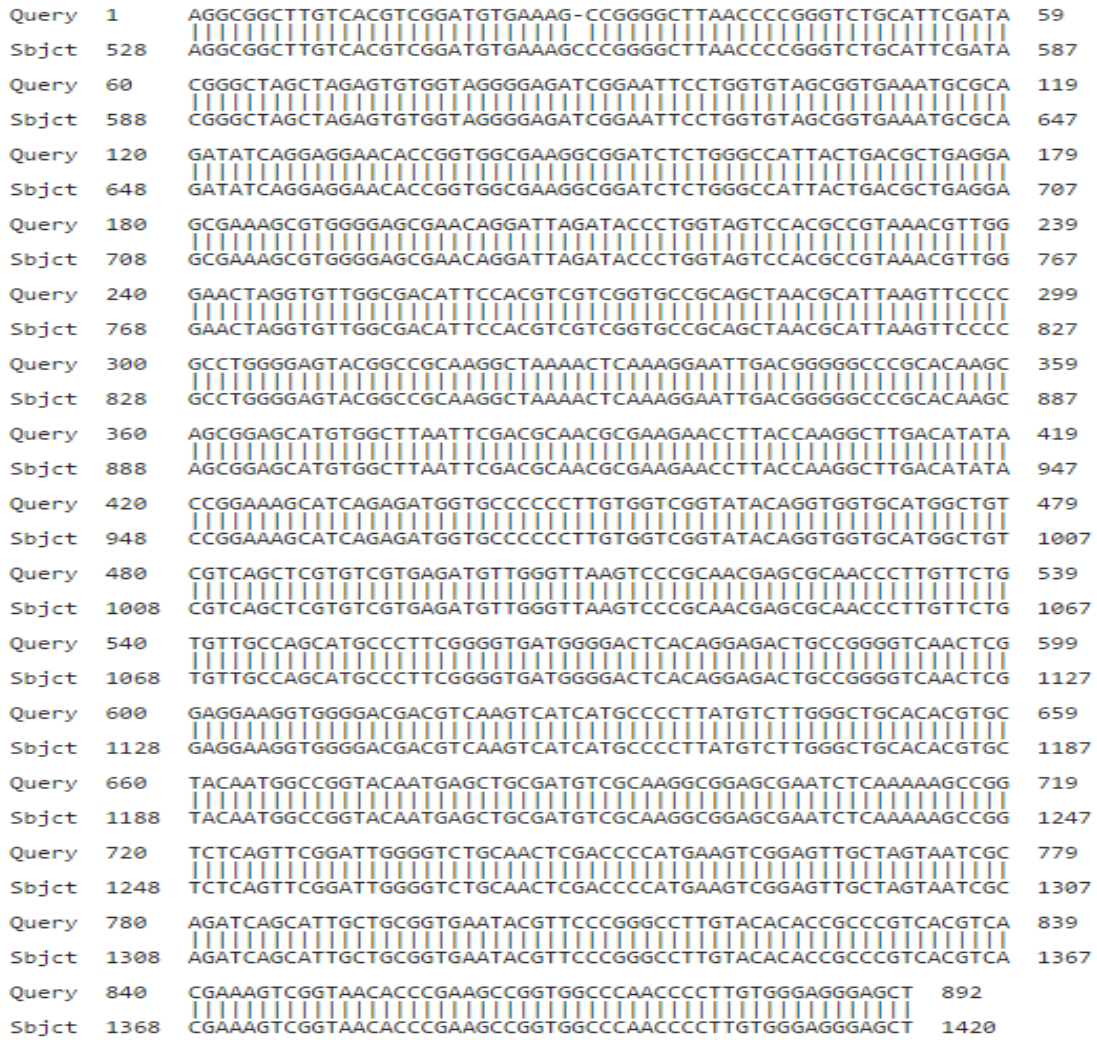


Figure 3.12: *Streptomyces verne* strain: NBRC 3112

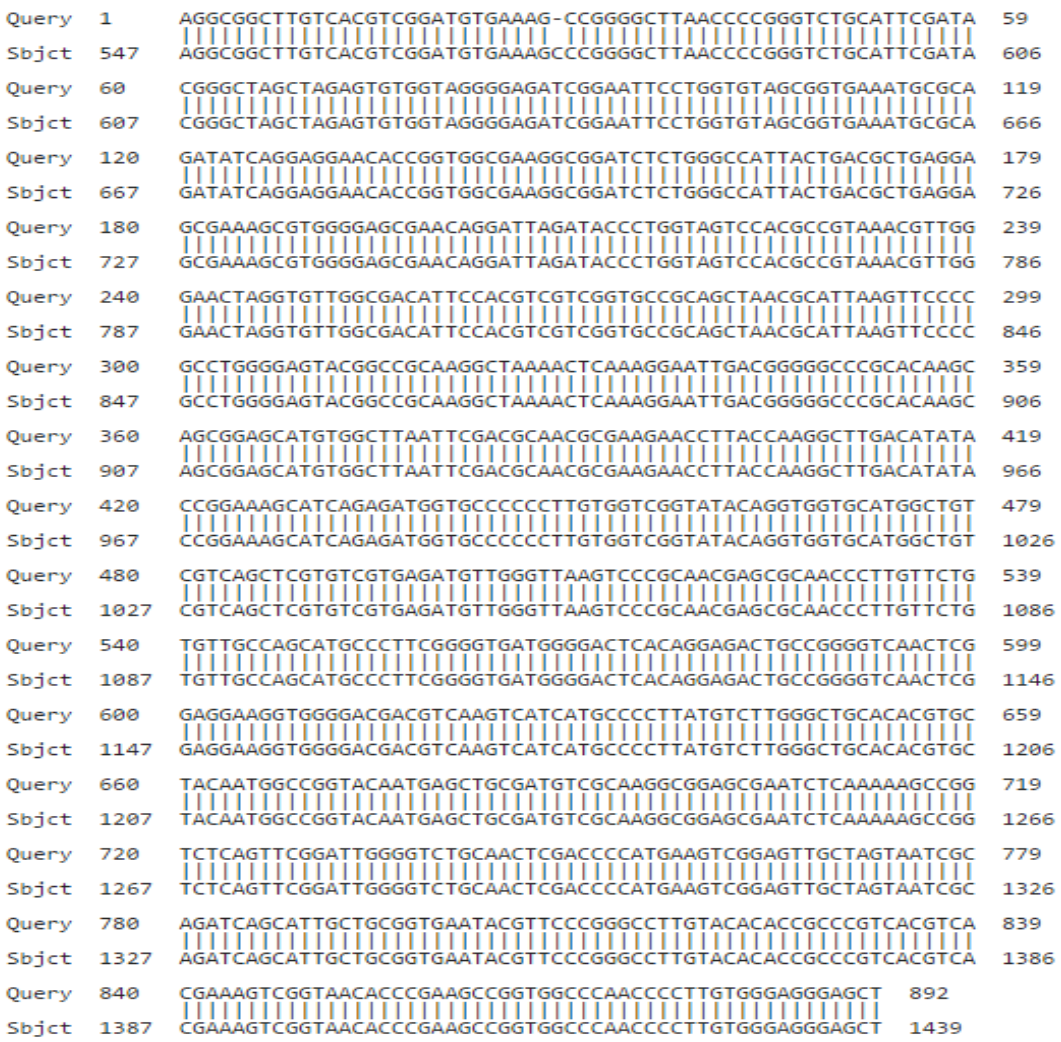


Figure 3.13: *Streptomyces* sp NBRC 3111

3.3 Discussion

The aims of the study were to examine terrestrial and aquatic invertebrates for the presence of antibiotic producing microorganisms in the hope that organisms producing novel compounds could be isolated. The results showed that it was possible to isolate antibiotic producing strains from organisms from both sources. In the last two decades, the rate of discovery of novel compounds from terrestrial microorganisms has declined significantly. Recently researchers have begun investigating unconventional sources due to lack of research within those particular areas. This includes terrestrial invertebrates (Madden *et al*., 2013). Antibiotic producers have previously been reported in terrestrial invertebrates. Antibiotic producing streptomycetes have been reported in wasps but these were from a different species of wasp which is thought to produce antibiotics (not yet characterized) that the wasp smears over brood cells prior to oviposition to protect eggs from a fungal parasite (Kaltenpoth *et al*., 2005). Antagonistic activity was reported in a new strain of *Streptomyces tritolerans sp*. isolated from earthworm gut. Ethyl acetate extract of these strain showed high antagonistic activity against *Aspergillus flavus, Fusarium udum, Fusarium oxysporum, Rhizoctonia solani, Alternaria alternate* and *Colletotricum capscii* (Aruna *et al*., 2008). Isolation of a *Pseudomonas aeruginosa* strain producing an uncharacterized antimicrobial agent with the broad spectrum activity against *E. coli* ATCC 25922, *S. aureus* ATCC 25923, *B. subtilis* ATCC 465,*E. faecalis* ATCC 29737, *Candida albicans* ATCC 10231 and *K. pneumoniae* ATCC 10031, it has been reported from the digestive system of an Iranian endemic slug, *Parmacella iberica* (Ebrahimipour *et al*., 2011). Also, antibiotic producers have been reported in marin invertebrates, antibiotic producing bacterial strains (*Bacillus megaterium, Vibrio sp., Idiomarina baltica, Dietzia sp., Bacillus marisflavi, Staphylococcus equorum, Salinicoccus roseus, Bacillus sp., Marinobacter sp., Bacterium K54, Pseudomonas sp., Ruegeria sp., Vibrio parahaemolyticus, Bacillus subtilis , Vibrio fortis* and *Vibrio sp*.) associated with four species of sponges (*Echinodictyum* sp., *Spongia* sp., *Sigmadocia fibulatus* and *Mycale mannarensis*) were isolated from the Tuticorin coast, Gulf of Mannar region. The strain of *Bacillus* sp. was found to produce a highly active compound against *B subtilis, E coli* and *Candida albicans* but the structure of this compound has not been characterized (Anand *et al*., 2006). Three compounds (cottoquinazolines D, cottoquinazolines B and

cottoquinazolines C) were isolated from coral-associated fungus *Aspergillus versicolor* LCJ-5-4 (soft coral *Cladiella* sp., South China Sea). These compounds were evaluated in vitro for cytotoxicity against Hela (cervical carcinoma) and P388 (murine leukemia) cells line and for antimicrobial activity against *E. coli, S. aureus, Enterobacter aerogenes, B. subtilis,* and *Candida albicans.* Cottoquinazolines D exhibited moderate antifungal activity against *Candida albicans* while other compounds did not show antimicrobial activity, the Three compounds showed no cytotoxicity against the tested Hela and P388 cell lines (IC_{50} > 50 μM) (Zhuang *et al*., 2011). Mayamycin was extracted from a *Streptomyces* sp. strain HB202 isolated from the Marine Sponge *Halichondria panacea* from the Baltic Sea showed activity against several bacteria including antibiotic-resistant strains (*B. subtilis* (DSM 347, *Brevibacterium epidermidis* (DSM 20660), *Dermabacter hominis* (DSM 7083), *K. pneumoniae* (ATCC 700603; ESBL) *Propionibacterium acnes* (DSM 1897), *P. aeruginosa* (DSM 50071), *S. aureus* (ATCC 12600), *S. aureus* (ATCC 33593; MRSA), *Staphylococcus epidermidis* (DSM 20044) , *Staphylococcus lentus* (DSM 6672) and *Xanthomonas campestris* (DSM 2405)) and exhibited potent cytotoxic activity against eight human cancer cell lines (HepG2 (hepatocellular carcinoma), HT-29 (colon adenocarcinoma), GXF251L (gastric cancer) , LXF529L (non-small-cell lung cancer), MAXF401NL (mammary cancer), MEXF462NL (melanoma cancer), PAXF1657L (pancreatic cancer) and RXF486L (renal cancer) (Schneemann *et al*., 2010). As far as the author is aware none of these has been commercially exploited so far, however, the results presented here support invertebrates as sources of antibiotic producing microorganisms which may give useful antibiotics.

In this study and during the primary screening program, a total of 125 microbial isolates from 35 samples of different invertebrates were initially screened, by the overly-agar technique and agar-streak method for the ability to produce antibacterial metabolites. Whether the microorganisms were present in the invertebrates having been obtained from the environment e.g. from food or washed in from surrounding soils or whether they were there as part of a symbiotic or other association needs to be confirmed by further studies such as by trying to isolate organisms from same invertebrates to prove that producing organism were present in each time. The results revealed that 47 of the isolates (37.6 %) were active against the Gram positive and

Gram negative bacteria tested. This percentage seems encouraging as compared to some reports which quote a rather lower percent of active isolates from the total number isolated. For example (Zheng *et al*., 2005) also reported that 8 out of 29 strains, representing 28% of the isolates considered in their study produced antimicrobial activity against at least one of the test microorganisms. (Ivanova *et al*., 1998) reported that, out of the 491 bacteria isolated from different marine sources, 26% of the isolates were active. In contrast, (Motta *et al*., 2004) found 86.6% active isolates out of 86 isolates from aquatic environment of Brazilian Amazon Basin.
The antibacterial assay for isolates used two commonly used laboratory techniques, namely the overlay–agar technique and agar-streak method for comparative purposes and reliability of results. However, during this study, the streak assay technique showed more conclusive results and was relatively easier to use for primary screening of the biologically active metabolites of the isolates.
Antimicrobial susceptibility has been evaluated by different qualitative and/or quantitative methods, but a reliable standardization for comparison is frequently missing (Wong *et al*., 2016). However, most of the common antimicrobial susceptibility assay methods require the purification of the antimicrobial substance or the use of an enriched fraction of it. Such a purified compound / fraction can then be tested by diffusion or dilution methods or other modern techniques (Finger *et al*., 2013, Wang *et al*., 2014b)
Although 5 test organisms were used in the assay program representing both Gram positive (2) and Gram negative bacteria (3), most of the active isolates had activity against S. *aureus* which was the most sensitive test organism for all isolates (Table 3.1) since about 31 isolates (~65.9 % of the total) were found to be active by showing a diameter of inhibition zone more than 5 mm. The limited number of test organisms, has previously been acceptable in primary screening programs (Svahn *et al*., 2012, Compaore *et al*., 2016a) for supplying sufficient and convenient results.

This work represents just one ring of an endless chain of searching for novel antibiotics from nonconventional or uncovered sources and not fully studied (invertebrates). Fungi isolated from marine invertebrates are of substantial significance as novel promising sources of distinctive secondary metabolites with important biomedical potential (Yue *et al*., 2015). The estimated 100,000 species of fungi that are known today make up only about 10 percent of all fungal species that

are predicted to exist, implying that fungi are an unmapped and untapped source of secondary metabolites with bioactive potential (Scheffers *et al*., 2012). Thousands of *Penicillium* isolates have probably been screened in bioprospecting programs since the discovery of penicillin but new bioactive metabolites still continue to be discovered from these fungi (Larsen *et al*., 2007, Ge *et al*., 2008, Takahashi and Lucas, 2008) indicating their current importance as sources of novel bioactive molecules to be used by pharmaceutical industry (Petit *et al*., 2009).

Comparative antibiotic production using different media

The effect of carbon and nitrogen source has been the focal point of study for industries as well as research groups. In this study seven types of carbon and nitrogen containing media were tested for a maximizing the antibiotic production (SNB, SAB, GNB, YMB, YEB, TSB and AIB). Individual cultures of Es1 on YMB, YEB and AIB media, showed that the media which contained yeast extract as carbon and nitrogen sources, gave large zone sizes. Also, cultivation of Es1 on AIB gave good growth and antibiotic production; it contained carbon and nitrogen sources (glycerol as source of carbon and L-Asparagine as nitrogen source). However use of SNB and SAB, was not promising for getting better activity (inhibition less than 5 mm) when compared the YEB, YMB and AIB.

Mycelium growth (biomass) of Es1 was similar to other media but with no appreciable antibiotic production in case of using mixed medium (SNB+NB; 2:1 and SAB+NB; 2:1 v/v), this may probably referred to some sort innate antagonism occurred between starch- containing media fortified with different nitrogen sources. (Tawiah *et al*., 2012) noted that asparagine gave better yield for antibiotic production by *P. aeruginosa* rather than inorganic nitrogen sources such as sodium and potassium nitrates or ammonium salts.

(Lee *et al*., 1997) reported that ammonium sulfate was the best with respect to formation of rapamycin by *S. hygroscopicus* comparable to six non-amino acid nitrogen compounds were examined. Potassium nitrate, followed by L-asparagine, sodium nitrate, glycine, DL-aspartic acid and L-tryptophan were the best nitrogen sources for the production of penitrem B by *P. aurantiogriseum* (Surekha and Reddy, 1992). *Streptomyces* spp. TN97 strain showed that the highest biological activities were also obtained when glycerol or fructose at 1% (w/v) was added in tryptic soy

broth (TSB) (Mehdi *et al*., 2006). (Compaore *et al*., 2016a) reported that the best carbon sources for production of antibacterial compounds appeared to be starch and glucose for *Aspergillus flavus* and starch and sucrose for *Penicillium citrinum.*

Identification of biologically active isolates by DNA plus PCR

Different kits were used for DNA extraction the most satisfactory kit was *FastDNA* spin kit for both bacteria and fungi with acceptable purity and concentration for downstream application e.g. PCR and sequencing. Two genes were targeted for identification of the isolates, 16S for bacteria and 18S rDNA for fungi, Forty-two (89%) of the 47 isolates were fungi while the remaining 5 (11%) were *Streptomyces* spp.

Although the initial methods were chosen to specifically isolate actinomycetes, there were only 5 *Streptomyces* spp. among the isolates and the majority were fungi, mostly *Penicillium* spp. Initially some of the isolated proved to be mixed cultures containing both a fungus and a streptomycete. This was demonstrated using whole genome sequencing using a MiSeq (data not shown). Although the antimicrobial activity could have been produced by the streptomycete or production could have been promoted in the mixed cultures as previously demonstrated (Schroeckh *et al*., 2009, Moody, 2014, Lim *et al*., 2012, Valayil, 2016), further subculturing led to a pure culture of the fungi which still produced the antimicrobial activity. The use of media for actinomycetes for growing the fungi may have enhanced the production of the antimicrobials or may have activated silent genes.

Three strains (Es1, M1 11 and La1a) were also sent for identification to an external source for confirmation and identification under species status, the result of 18S rDNA sequencing for this isolates were identified as *Penicillium* species. For more identification of the under species status, the isolates were sent to NICMB Limited Company, to sequence ITS gene to distinguish between species because it has recently been accepted as the official sequence for identification of fungi (Schoch *et al*., 2012). The results identified M1 11 as *P. chrysogenum* but Es1 and La1a identified just as *Penicillium* spp. Depending on the obtained data, this level of identification of the spp. regarding the strains La1a and Es1, may be attributed to absence of sequencing ITS data in the gene bank or, may be, it is a new species and not discovered before.

Following completion of the work it was discovered that the ITS region has limitations for determining the species of *Penicillium*. The β-tubulin (BenA) gene has been proposed as a secondary identification marker although it too has its limitations (Visagie *et al*., 2014).
Also, the use of BLAST searches against the Genbank database has problems as a large number of the sequences for *Penicillium* spp. have not been verified and some have been incorrectly identified (Kõljalg *et al*., 2005).

The limitation of this part of the study were relatively small number of invertebrates screened but the result indicated that a larger study would be worthwhile.

Chapter 4

Production and extraction of antibiotics

4.1 Introduction

4.1.1 Production

As bioactive metabolites are typically generated in small quantities and production depends on the species and the growth parameters, optimisation of these factors is essential for maximum quantity and purity of secondary metabolites (Merlin *et al*., 2013). Research in finding newer antibiotics and increasing the productivity of such agents has been a very important activity (Sundaramoorthi *et al*., 2011, Retinowati *et al*., 2010).

Development of an efficient large scale production process to produce secondary metabolites by microorganisms requires examination of a diverse array of species-specific features including nutritional factors such as carbon and nitrogen sources. These play key roles as structural and energy compounds in a cell (see chapter 3). Several cultivation parameters e.g. pH, incubation period, aeration rate and temperature play a significant role in the production of bioactive metabolites (Compaore *et al*., 2016a). A slight change in the culture medium will not only impact on the quantity of certain compounds but also the general metabolic profile of microorganisms (Scherlach *et al*., 2009, Wang *et al*., 2011).

In the industrial setting, optimization of growing conditions can lead to the significantly increased production of a compound of interest. The manipulation of environment and nutrition has been shown to have substantial impacts on the quantity and diversity of secondary metabolite production (VanderMolen *et al*., 2013). Compaore *et al*. (2016a) reported that the antibacterial compound production started on the third day by *Aspergillus flavus* and *Penicillium citrinum,* and the highest antibacterial compound production was between 7-9 days during the stationary phase. Tawiah *et al*., (2012) found that nine days incubation period was optimum for maximum antibacterial activity by *Pseudomonas aeruginosa*, an indication of maximum antibiotic production, after which there was no significant increase. Whilst, Compaoré *et al*. (2016b) reported that production of antimicrobial compound by

Aspergillus fumigatus started on the fourth day of fermentation and the optimum incubation period for the higher production of bioactive compound was 6-8 days (pH 7, temperature 37 °C with rotary shaker 150 rpm).

4.1.2 Extraction

The initial step in isolating biologically active compounds is referred to as extraction. The compound extracted from any natural source as fungi, bacteria and plant is dependent on factors such as solvent type, extraction method and cultivation features. Methods used will depend on whether the desired compounds are endocellular or excreted into the medium. Extraction methods are a main consideration as they relate to efficiency in getting the highest yields of natural compounds from natural sources (Hip *et al.*, 2009).

To obtained new bioactive compounds from natural products, fractionation is usually required using multiple solvents with differing polarity (Cowan, 1999; Dinan *et al.*, 2001). Bioactive compounds are extracted from natural sources in small quantities and are present as conjugates or mixtures in extracts which may therefore require lengthy and intense purification processes. (Joana *et al*., 2013).
Various techniques have been established to enhance the extraction of bioactive compounds from natural sources. The most common technique is referred to as Solvent Extraction (SE) used to obtain specific compounds from various materials such sediments, soil, bacteria, fungi and various plants (El Hattab *et al*., 2007; Plaza *et al*., 2010). The samples obtained are fed through various filtration processes such as centrifugation to filter unnecessary residues. Organic solvents have been used through time to extract bioactive compound from natural sources. From among the most common are hexane, ether, chloroform, acetonitrile, ethyl acetate, benzene and ethanol which are commonly used in various ratios with water. (Tokuoka *et al*.,2010, Plaza *et al*., 2010). These organic solvents can be used in the extraction of polar and non-polar bioactive compounds like fatty acids, phenols, aromatic hydrocarbons, alkaloids and the like (Szentmihalyi *et al*., 2002, El Hattab *et al*., 2007, Li *et al*., 2006). Nevertheless, extra care should be practiced when handling certain solvents as they are harmful to not the person handling them but also to the environment; furthermore, it is important to note that extraction processes can be arduous (Miron *et al*., 2011, Li *et al*., 2006).

Solvent Extraction is one of the most convenient techniques compared to others as it is cheaper and easier to run, especially on an industrial scale if solvent recovery and reuse is practiced. Although, this technique uses potentially toxic and inflammable solvents to operate, involves an evaporation step and requires large quantity of solvents and is time consuming. Additionally, there is a risk of bioactive compound thermal degradation due to the hot temperature of solvents used during the long times of extraction. In general, extraction methods are constantly improving through the development of existing/new techniques such as Ultrasonic assisted extraction (UAE), Microwave-assisted extraction, Subcritical fluid extraction and Accelerated solvent extraction has been promoted (Szentmihalyi *et al*., 2002, Bendicho *et al*., 2012); The ultimate objective of these developments are to shorten the lengthy extraction time and energy consumed which would ultimately result in lowering costs. Therefore these techniques are sustainable in that they also minimize any harm to the persons handling and the environment, minimizing costs (Armenta *et al*., 2015). From the mentioned techniques UAE is the most efficient as it is safer to handle and economically advantageous and reproducible because the process involves atmospheric environments and ambient temperature (Soria *et al*., 2010, Vieira *et al*., 2013).

The aims of this part of the study were to culture one isolate (Es1) on one chosen medium (AIB), to extract the active compounds from culture medium, optimise maximum antibiotic yields using different solvent extraction systems and to test the spectrum of activity of the antibiotics against a range of test organisms. The spectrum was also determined for all antibiotic producing organisms isolated. The best three organisms were chosen on the basis of their activity and large scale culture used to prepare sufficient material for subsequent characterization.

4.2 Results

4.2.1 Production, Extraction of antibiotics from broth medium

4.2.1.1 Production of the antibiotics

Isolate Es1was heavily inoculated into 250 ml sterile AIB in 1 L conical flasks and incubated for 7 d as described in section 2.12.1. The medium was filtered and 1 ml was placed in wells made in seeded MHA plates inoculated with a lawn of test organisms. The results (Table 4.1) showed that activity was secreted into the culture medium and both *E. coli* and *S. aureus* were inhibited.

Table 4.1: Antibacterial activity result of filtered AIB of isolated Es1 against test organisms

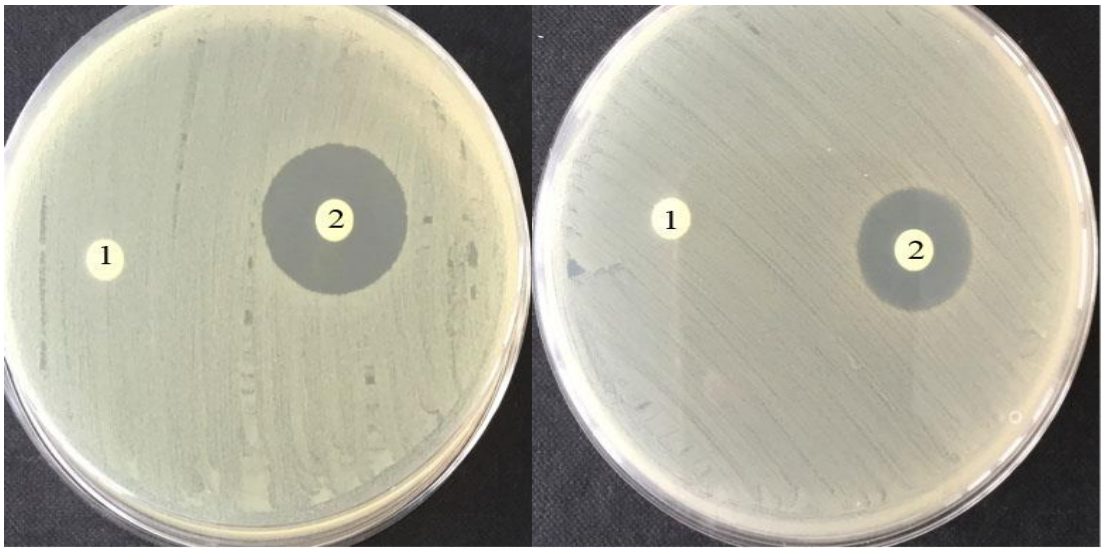
Test organism	Inhibition zone diam. mm
E.coli ATCC10536	12
S. aureus ATCC 25923	14

4.2.1.2 Extraction of antibiotics

The filtered medium was extracted with ethyl acetate; the organic layer was then separated and evaporated by rotary evaporation. The crude extract obtained was dissolved in ethyl acetate, and then tested using the paper disc diffusion method. The aqueous layer was tested by the agar well diffusion method. The results are shown in Table 4.2 and Figure 4.1. The organic layer showed antimicrobial activity but there was no residual activity in the aqueous layer. Mycelium extracts prepared with acetone had a much lower activity (Table 4.3) showing that most of the activity was secreted into the medium (the controls were paper discs loaded with acetone solvent).

Table 4.2: Inhibitory activity of organic layer and aqueous layer of extracts from isolate Es1 against test organisms

Test organism	Inhibition zone diameter mm		
	Es1 organic layer	Es1 aqueous layer	Ethyl acetate (control)
E.coli ATCC10536	12 mm	-	-
S. aureus ATCC 25923	18 mm	-	-



A) B)

Figure 4.1: Inhibitory activity of paper disc loaded with Es1 extracts of medium (A) S. *aureus* ATCC 25923 and (B) *E. coli* ATCC10536*;* (1) ethyl acetate control, (2) Es1 organic layer

Table 4.3: Antibacterial activity of the Es1 mycelium extract prepared with acetone against *S. aureus* and *E.coli* after 12 d of incubation in AIB.

Test organism	Inhibition zone diameter mm	
	Es1 mycelium extract	Acetone (control)
E.coli ATCC10536	3 mm	-
S. aureus ATCC 25923	4 mm	-

- No inhibition zone

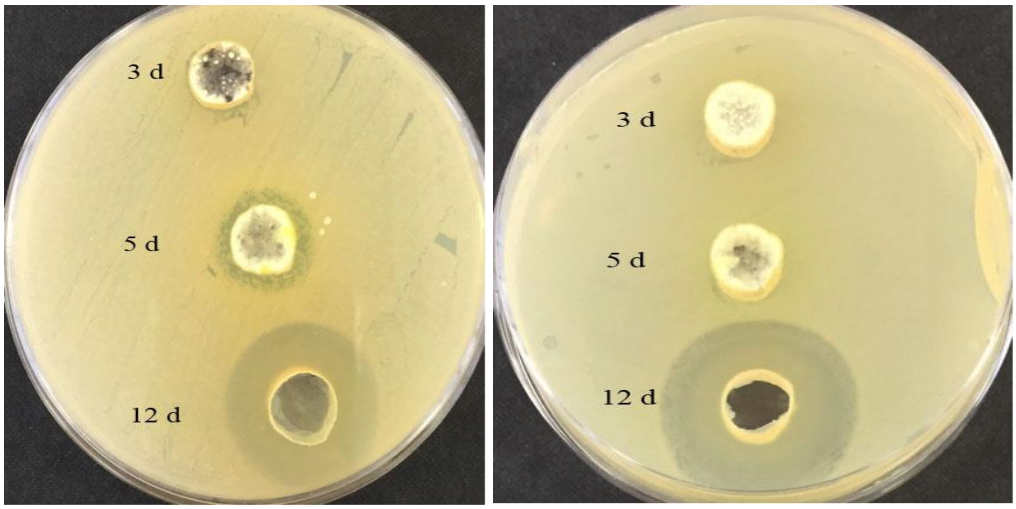
4.2.1.3 Effects of incubation time on yield of antibiotics

Isolate Es1 was grown in 300 ml portions of AIB in 1L conical flasks at 28±2ºC and 120 rpm for 3, 7 and 12 d. Broth samples were filtered through cotton wool to remove mycelium and tested for their antimicrobial activity. The results are shown in Table 4.4 and Figure 4.2. The results showed that slight activity against *S. aureus* was apparent after 5 d but maximum activity against both organisms was obtained after 12 d.

Table 4.4: Antibacterial activity of the Es1 extracts after three, five and twelve days of incubation on AIB

Test organism	**Antibiotic activity after incubation time d**		
	3	**5**	**12**
S. aureus ATCC 25923	-	4 mm	17 mm
E.coli ATCC10536	-	-	19 mm

Inhibition zone diameter mm, - No inhibition zone



(A) (B)

Figure 4.2: Effects of incubation time in AIB on antimicrobial activity of extracts from isolate Es1

Inhibition zone activity around the wells in seeded MHA plate with *S. aureus* ATCC 25923 and *E.coli* ATCC10536 and filled with 1 ml of filtered AIB after 3, 5 and 12 d incubation. (A) MHA plate seeded with *S. aureus* ATCC 25923, (B) MHA plate seeded with *E.coli* ATCC10536.

4.2.2 Production, Extraction of antibiotics from solid medium

4.2.2.1 Extraction of antibiotic

Strain Es1 was grown on two plates of AIA at 28°C for 7 d. The agar with the organism was then cut up into small pieces and placed into a flask with 40 ml ethyl acetate. Two different methods were used to extract the antibiotic, homogenising the agar with the ethyl acetate using a magnetic stirrer for 2 h and sonication for 1 h. The

results are shown in Table 4.5 and Figure 4.3.The sonication method gave better extraction with a higher antibacterial activity and also took less time.

Table 4.5: Antibiotic activity of extracts of isolate Es1 produced by different extraction methods

Isolate	Organism	Zone of inhibition mm Extraction method	
		Magnetic stirrer	Sonicator
Es1	*E.coli* ATCC10536	8	10
	S. aureus ATCC 25923	7	12 mm

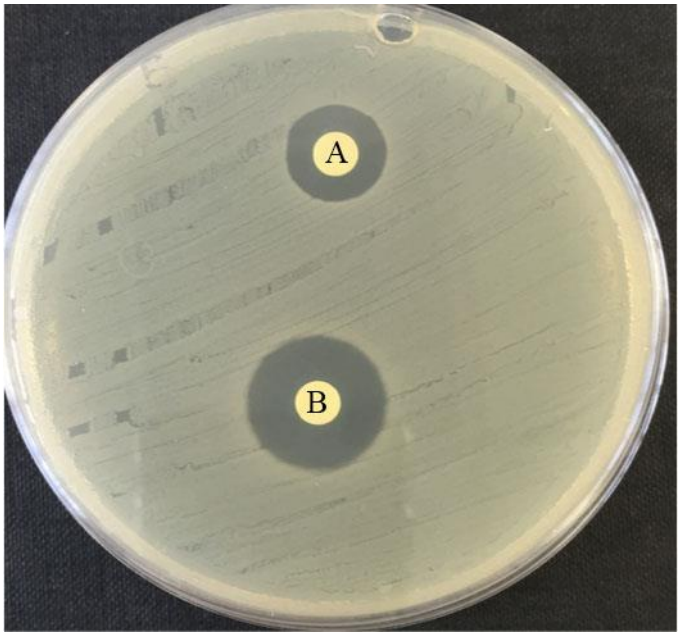


Figure 4.3: Antibiotic activity of extracts produced by different methods,
Plate of MHA seeded with *S. aureus* ATCC 25923 showing clear inhibition zone, (A) magnetic stirrer method, (B) sonicator method.

4.2.2.2 Effects of inoculation method on yield of antibiotics

Average clear zones of antimicrobial activity of extracts were determined using three different inoculation methods as described in section 2.12.4.2 and the results are shown in Table 4.6. The results show that the zigzag method gave the largest inhibition zones.

Table 4.6: Effects of inoculation method on antibiotic activity of extracts

Strain code	Test organism	Zone of inhibition mm Inoculation Method		
		Zigzag	**Streak**	**Individual colonies**
Es1	*E. coli* ATCC10536	11 mm	9 mm	10 mm
	S. aureus ATCC 25923	13 mm	10 mm	11 mm

4.2.2.3 Scale up number of plates extracted

Six agar plates of Es1 incubated for 7 d were cut to small pieces, placed in a flask, 120 ml ethyl acetate added and sonicated for 1 h then filtered. For scale up 14 plates were used, the agar was cut into small pieces and put in beaker. Ethyl acetate (280 ml) was added and sonicated for 1 h then filtered, Antibiotic activity was determined as before and the results are shown in Table 4.7. Activity was lower when 14 plates were used despite the volume of ethyl acetate being increased in proportion to the number of plates.

Table 4.7: Effects of scaling up number of plates extracted on antimicrobial activity

Strain code	Test organism	Zone of inhibition mm Number of Agar plates	
		6	**14**
Es1	*E.coli* ATCC10536	15	10
	S. aureus ATCC 25923	16	9

4.2.2.4 Time of incubation

Isolate Es1 was inoculated onto 6 plates of AIA and incubated at 28°C for 7 d and a further 6 plates incubated for 21 d. Antibiotics were extracted and assayed as before and the results are shown in Table 4.8. Activity was higher after 21 d and all subsequent incubations were for 21 d unless otherwise stated.

Table 4.8: Effects of incubation time on antimicrobial activity of extracts from isolate Es1

Strain code	**Test organism**	**Zone of inhibition mm Time of incubation d**	
		7	**21**
Es1	*E.coli* ATCC10536	12	16
	S. aureus ATCC 25923	13	19

4.2.2.5 Use of different solvents for antibiotic extraction

Cultures of isolate Es1 grown on AIA were cut up into small pieces and extracted with 4 different solvents, filtered and assayed for antimicrobial activity. The results are shown in Figure 4.4, ethyl acetate gave the highest activity followed by acetone. Chloroform and chloroform-ethyl acetate gave the lowest activities. Ethyl acetate was used subsequently for extraction of the antibiotic activity.

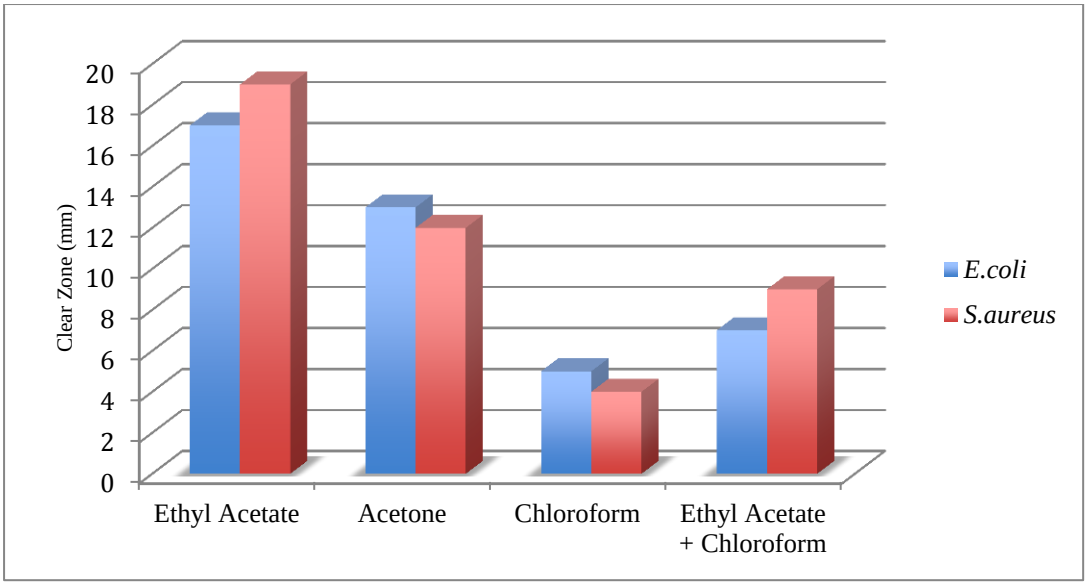


Figure 4.4: Comparative analysis of solvent used for extraction of the antibiotic from cultures of the isolate Es1 grown on AIA.

4.2.2.6 Antibacterial activity of ethyl acetate extracts of other isolates

All isolates that showed antibacterial activity in the primary screen were grown on AIA at 28°C for 21 d, then 6 plates of each isolate were extracted with 120 ml ethyl acetate and antimicrobial activity determined as before and the results are shown in Table 4.9. The results showed that some isolates that showed some antibacterial activity in the primary screen had no inhibition zones for the ethyl acetate extracts. Only three extracts had inhibition zones against *E. faecium* (La1a, La2 and W12) and one isolate against *E. faecalis* (W12). Most of the extracts isolates had activity against *S. aureus2* which was the most sensitive test organism for the extracts, since about 25 extracts isolates (~53% of the total) were found to be active by showing inhibition zones (between 4-18 mm). Twenty-two of the extracts isolates (48.8% of the total) showed activity against MRSA and gave inhibition zones of 2-13 mm, while 24 extracts (~51% of the total) showed activity against ESBL *E.coli* with inhibition zones of 2-13 mm. Extracts from isolates Es1, M1 11 and La1a (shown in bold in Table 4.9) showed the best antibacterial activity compared to the other isolates and were chosen for further study. The appearance of these three organisms are shown in Figure 4.5. All three had similar colonies with a grey powdery appearance. Isolate Es1 had a filamentous colony edge and yellow pigmentation of the medium. Isolates M1 11 and La1a had more entire edges to the colony and M1 11 had a folded colony center.

Table 4.9: Inhibition zones of ethyl acetate extracts of cultures of microorganisms isolated from invertebrates.

Isolate	Zone of Inhibition (mm) against test organism											
	Gram-positive						Gram-negative					Yeast
	B. c	S. a	S. a 2	MRSA	E. f'm	E. f's	*E. coli*	ESBL *E. coli*	P.a	P.f	K. P	S. c
Sa1	0	0	0	0	0	0	0	0	0	0	0	0
Sa2	0	0	0	0	0	0	0	0	0	0	0	0
La1a	**9**	**11**	**14**	**9**	**2**	**0**	**12**	**9**	**9**	**7**	**0**	**5**
La2	7	7	8	5	1	0	8	5	7	6	0	6
La4	4	5	6	6	0	0	5	7	6	4	0	4
La5	7	8	11	4	0	0	8	6	8	6	0	7
Es1	**9**	**14**	**18**	**13**	**0**	**0**	**18**	**13**	**12**	**1**	**0**	**10**
Es2	0	0	0	0	0	0	0	0	0	0	0	0
Es5	0	0	0	0	0	0	0	0	0	0	0	0
Es9	0	0	4	0	0	0	0	0	0	0	0	0
M1 2	9	10	12	8	0	0	10	10	3	6	6	0
M1 3	10	10	13	12	0	0	3	10	6	9	8	0
M1 4	10	9	13	11	0	0	10	12	6	7	7	0
M1 8	8	9	10	11	0	0	9	9	0	12	1	0
M1 10	9	10	12	12	0	0	10	10	3	7	7	0
M1 11	**10**	**14**	**15**	**12**	**0**	**0**	**16**	**10**	**6**	**8**	**6**	**0**
M1 13	8	8	11	12	0	0	7	8	3	6	5	0
M1 14	9	9	9	11	0	0	10	9	5	6	5	0
M1 15	8	10	12	12	0	0	9	10	4	7	5	0
M1 16	8	9	13	11	0	0	10	10	6	6	4	0
M118	8	9	10	8	0	0	9	0	2	6	3	0
M1 20	4	6	12	12	0	0	10	11	1	3	6	16
M1 22	6	7	11	8	0	0	8	7	2	4	5	0
M35 1	0	5	1	0	0	0	0	2	0	1	1	9
M35 4	0	3	0	0	0	0	0	2	0	0	0	8
M35 5	9	8	9	7	0	0	8	5	2	3	3	0
M35 9	2	15	6	8	0	0	7	7	1	6	7	20
M35 11	0	3	0	0	0	0	0	0	0	1	1	6
Pp1	0	0	0	0	0	0	0	0	0	0	0	0
Pp2	0	0	0	0	0	0	0	0	0	0	0	0
Pp3	0	0	0	0	0	0	1	6	0	0	0	0
Wata1	0	0	0	0	0	0	0	0	0	0	0	10
Wata 2	0	0	0	0	0	0	0	0	0	0	0	0
W1	0	0	0	0	0	0	0	0	0	0	0	0
W2	0	0	0	0	0	0	0	0	0	0	0	0
W3	0	0	0	0	0	0	0	0	0	0	0	0
W6	0	0	0	0	0	0	0	0	0	0	0	0
W7	5	11	12	11	0	0	13	12	3	0	0	0
W9	0	0	0	0	0	0	0	0	0	0	0	0
W12	2	2	0	2	1	1	2	3	0	4	2	10
W13	0	0	0	0	0	0	0	0	0	0	0	0

Isolate	Zone of Inhibition (mm) against test organism											
	Gram-positive						Gram-negative					Yeast
	B. c	**S. a**	**S. a 2**	**MRSA**	**E. f'm**	**E. f's**	***E. coli***	**ESBL *E. coli***	**P.a**	**P.f**	**K. P**	**S. c**
Antw1	0	0	0	0	0	0	0	0	0	0	0	0
Antw2	0	0	0	0	0	0	0	0	0	0	0	0
Antw3	0	0	0	0	0	0	2	0	0	0	0	0
Antb4	0	0	0	0	0	0	1	0	0	0	0	0
S5	0	0	0	0	0	0	0	0	0	0	0	0
S6	0	0	0	0	0	0	0	0	0	0	0	0

Key,: B. c = *B. cereus*, S. a = *S. aureus*, S. a 2 = *S. aureus MOH1*, E. f'm = *E. faecium*, E. f's = *E. faecalis*, P. a = *P. aeruginosa*, P. f = *P. fluorescens*, K. p = *K. pneumonia*, S. c = *S. cerevisiae*.

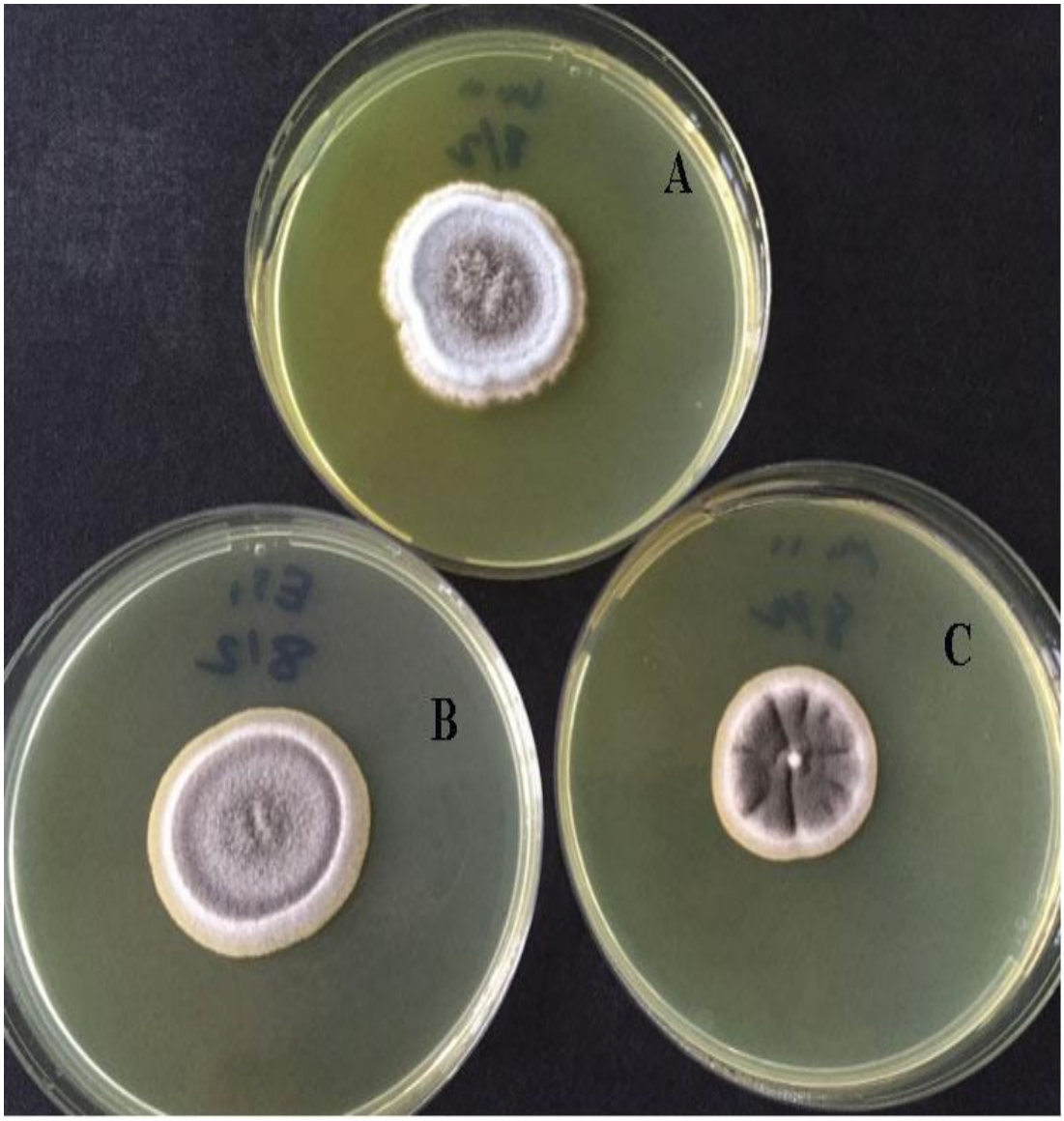


Figure 4.5: Single colonies of the three different isolates that showed best antibacterial activity on AIA
A: La1a

B: Es1

C: M1 11

4.2.3 Scale up of isolation of antibiotics produced by isolates Es1, M1 11, and La1a

Isolates Es1, M1-11 and La1a were each inoculated onto 300 AIA plates. The plates were incubated, extracted with ethyl acetate as before and the solvent removed by rotary evaporation. The crude extracts were dissolved in HPLC grade methanol giving approximately 200 ml of extracts of each isolate.

All extracts were filtered through 0.22µm filters to prevent damage to the HPLC columns. Dry weights were determined by weighing of crude amount from 2 ml/vial after evaporation and were 7.8 mg ml^{-1} for Es1, 6 mg $ml^{-1}$for M1-11 and 4.6 mg ml^{-1} for La1a.

All the extracts were tested for antibacterial activity before and after filtration. The result (Table 4.10 and Figure 4.6) showed that the extracts lost some activity on filtration but were still active.

Table 4.10: Activity of crude extracts before and after filtration through 0.22µm filters

Test organism	**Es1 extract**		**M1 11 extract**		**La1a extract**	
	Before	**After**	**Before**	**After**	**Before**	**After**
E. coli	19	14	19	12	13	9

Inhibition zone diameter mm, 10 µl of each extract was assayed.

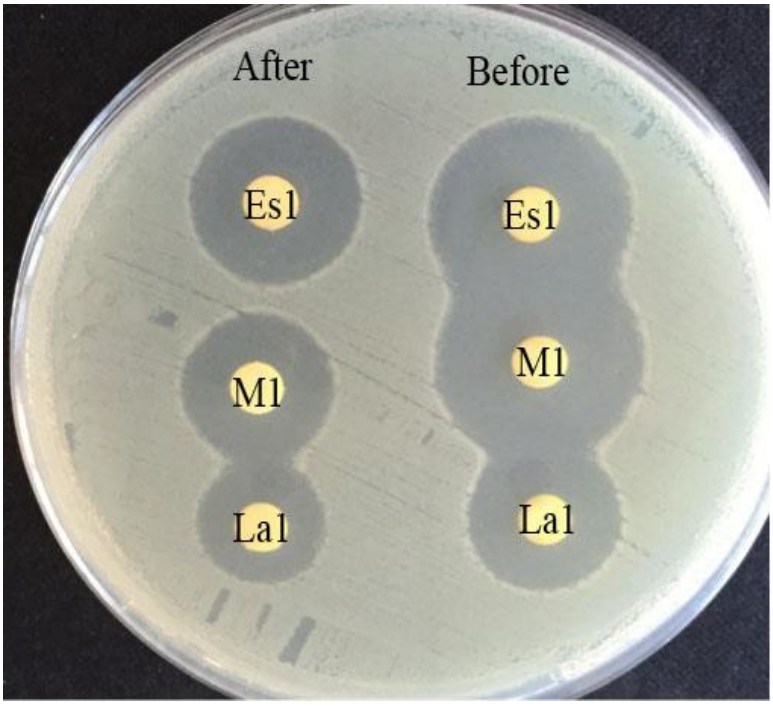


Figure 4.6: Seeded MHA plate with *E.coli* showing clear inhibition zones around paper discs loaded with 10 µl extracts before and after filtration (Es1, M1 11 and La1a)

4.2.4 Quantitative antibacterial activity of extracts

To test quantitative activity of the extracts, each extract was diluted to 1 mg ml^{-1}.and antibiotic activity determined (Table 4.11). The results showed that Es1 extracts were the most active followed by M1 11 then La1a extracts. The results also showed that *S. aureus* was more susceptible than *E. coli*.

Table 4.11: Comparison of antimicrobial activity of ethyl acetate extracts from different isolates

Test organism	**Zone of inhibition of extract mm**		
	Es1	**M1 11**	**La1a**
E. coli	13	13	10
S. aureus	21	15	12

All isolates were adjusted to 1 mg ml^{-1} dry weight and 10 µl assayed

4.2.5 Calibration Graph

Aliquots (5 µl, 10 µl, 15 µl, 20 µl, and 25 µl) of the 1 mg ml^{-1} extracts were loaded onto 6 mm filter paper disks and allowed to dry. Antibiotic activity was determined as before and the results are shown in Table 4.12 and Figures 4.7 and 4.8, the result showed the three extracts were more active against *S.aureus* than *E.coli*, the Es1 extract had the strongest activity followed by M1 11 and La1a respectively against both test organisms.

Table 4.12: Calibration curve of zone diameter vs concentration for crude extracts from three isolates

Test organism	Es1 isolate					M1 11 isolate					La1a isolate				
	5 µg	10 µg	15 µg	20 µg	25 µg	5 µg	10 µg	15 µg	20 µg	25 µg	5 µg	10 µg	15 µg	20 µg	25 µg
	Inhibition zone mm														
E. coli	10	13	14	15	15	9	12	13	13	13	5	10	11	11	11
S. aureus	18	21	22	23	23	9	12	13	16	17	7	12	13	15	15

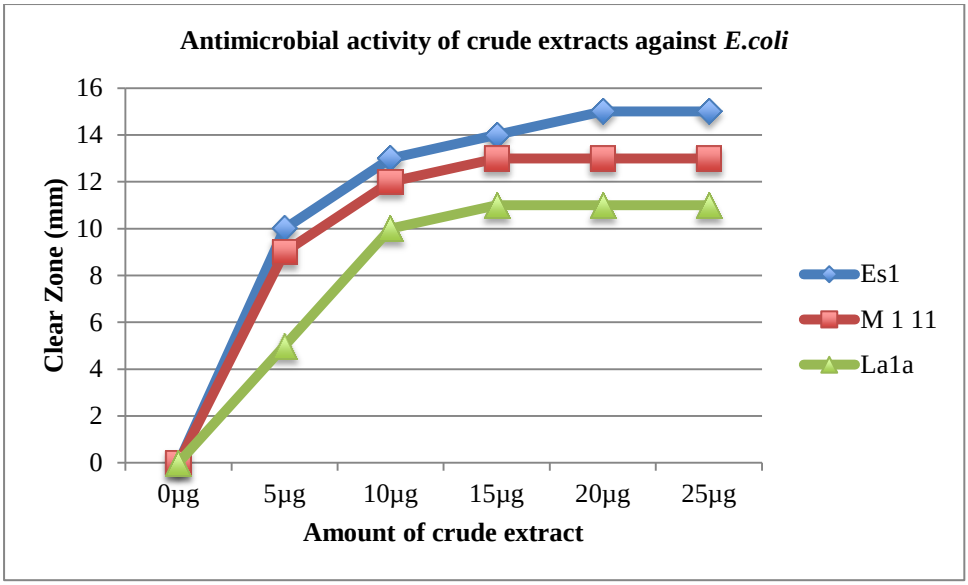


Figure 4.7: calibration curve for amount of antibiotic vs zone size
Antimicrobial activity of antibiotic extracts Es1, M1 11 and La1a against *E.coli*.

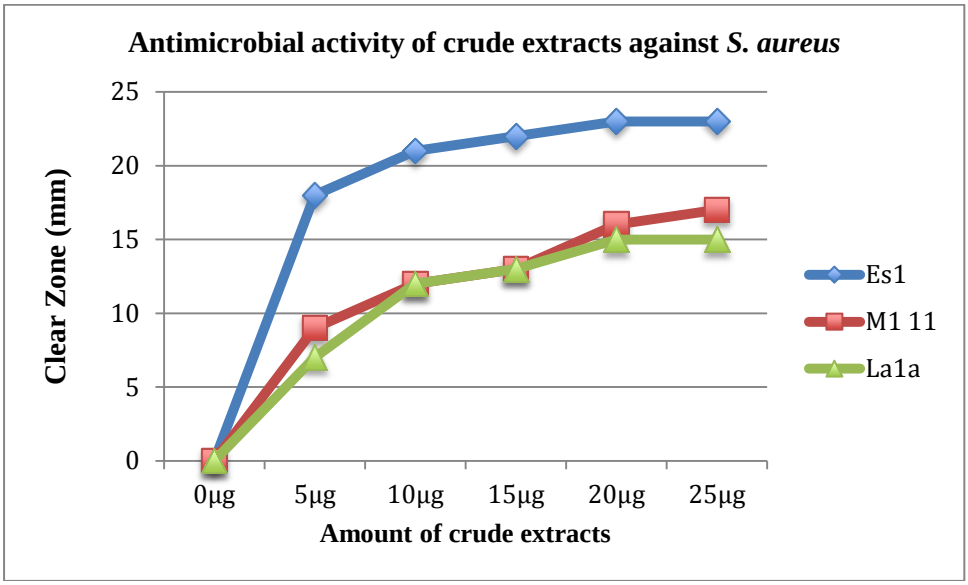


Figure 4.8: Calibration curve for antimicrobial activity of extracts from isolates Es1, M1 11 and La1a against *S. aureus.*

4.3 Discussion

Initially, production, extraction of the biologically active components synthesized by the isolate Es1 was by using broth medium. AIB medium, although designed for streptomycetes, was chosen as the growth medium as it had given good growth. The pH of this medium (7.0) was higher than standard media for fungi (pH 5-6) (Rahman *et al*., 2012, Gordon *et al*., 1947) and penicillin production in immobilized cells of *P. chrysogenum* was also optimum at an initial pH of 4 (Nagamune *et al*., 1988). Despite this the organism produced and an acceptable antibiotic yield.

The assay results were obtained after cultivation of the target organism (Es1) for 7 d, after which the culture was filtered then bio-assayed and showed activity against *E. coli* and *S. aureus* (inhibition zones of more than 5 mm). A further extraction step was accomplished using ethyl acetate: broth filtrate (1:1, v/v), after which, solvent was evaporated to dryness and the dark brown residual crude extract was eluted then bio-assayed with paper disc assay against the same organisms. The result showed more than 5 mm zones against test organisms; this result indicated that ethyl acetate succeeded in extracting the antimicrobial material from broth filtrate.

Time optimization for enhancement of antibiotics yield

Assay of the antibacterial activity in filtrates after 3, 5 and 12 days of incubation showed no activity after 3 days, after 5 days the bioactive material were only active against *S. aureus* (less than 5 mm). Extension of the incubation period up to 12 days improved the yield and spectrum of the antibiotic (more than 5 mm on both test organisms). The 12-day mycelium acetone extract also showed the highest antibiotic activity as tested by disc diffusion method on both test organisms (more 5 mm). (Compaore *et al*., 2016a) reported that the antibacterial compound production by the fungi they isolated was monitored over two weeks in synthetic broth. The course of antibacterial compound production and growth by the isolates showed the production started only after 48 h for both isolates. The highest rate of antimicrobial activity by the isolates was observed from days 7 up to 9 after its subculture into fermentation broth. There was an increase in the antibacterial activity produced by *Pseudomonas aeruginosa* metabolites up to the ninth day of incubation after which there was no significant increase (Tawiah *et al*., 2012). (Merlin *et al*., 2013) reported bioactive

metabolite production by endophytic fungi was monitored 15 days in synthetic broth, incubation period for 9 days was observed to be optimum for maximum production. (Tayung *et al*., 2011) also reported that incubation of endophytic fungi for 10–12 days was optimum for metabolite activity. In the present study, the maximum production of the antibiotic was at 12 days of incubation.

However, due to the lack of fermentation facilities in the School or in other Northern Universities to cultivate cultures of relatively large volumes of 10 L+ / run, it was decided to use an alternative method, that is the cultivation and extraction from a solid AIA medium to scale up the antibiotic(s) production. This made the fractionation more difficult as components from the cells and the culture medium would be co-extracted. This switch was necessary to save time and material and studies were performed to try to optimize the yield of antibiotics. Solid state culture has previously been used with success for a number of products including antibiotics using a variety of media (Pandey *et al*., 2000). For example penicillin has been produced from *P chrysogenum* using bagasse impregnated with growth medium (Barrios-Gonzalez *et al*., 1988). Svahn *et al*. (2015) reported the isolation of amphotericin from a fungus isolated from an extremely cold zone, they compared between extraction efficacy of solvents and procedure. The major extracts were, the filtrate of the liquid culture, the mycelial growth in the liquid culture, and the cultures growing on solid medium were also analyzed. They reported that, during amphotericin B production, cultures on solid medium proved to be a more reliable and favorable choice compared to liquid medium. Lamrani *et al*. (2008) similarly reported that various isolated fungi had increased antimicrobial production using media such as Sabouraud Agar, PDA (Potato Dextrose Agar), or MEA (Malt Extract Agar). These results agree with the results obtained here concerning the optimization of the antibiotic yield by growing the fungal spp. on solid medium.

Initial studies were on isolate Es1 but the same methods were also used for large-scale extraction from isolates M1-11 and La1a. Sonication and stirring methods for extraction into ethyl acetate were compared and the sonication method was better than stirring. (Kothari *et al*., 2012) also reported that sonication assisted extraction proved good at extracting antibacterial compounds.

Another pilot experiment was carried out in which Es1 inoculum was spread in three different ways (separate colony, streak, and zigzag), incubated and extracted. The highest yield was obtained from the zigzag method rather than separate colony and

streak methods. This was probably because the organism did not spread far from the inoculation sites and the zigzag method spread the organism out on the medium leading to better growth and increased yield. Initial experiments (data not shown) in which the whole plate was covered by spreading the inoculum gave a lower yield. It is likely that with the zig-zag method nutrients were able to diffuse into the colony from the uninoculated areas providing nutrients for antibiotic synthesis whilst not causing catabolite repression of secondary metabolite production.

Culture study for optimizing antibiotic yield on solid medium

1. Effect of incubation period on antibiotic yield

Two incubation periods (7 and 21 d) were used to cultivate isolate Es1, under the previously described conditions and the antibiotics produced were extracted of with ethyl acetate. For these experiments isolate Es1 was inoculated on plate surface and incubated at 28ºC, although some reports used a temperature of 25ºC for fungi (Petit *et al*., 2009). The results showed that the best incubation period was 21d and this was used in subsequent experiments. This taken with the results from broth medium suggest that the antibiotic activity was produced in the stationary phase of growth which is common for most fungi.

2. Effect of solvent type on extraction

Three organic solvents were utilized individually for extraction of the bioactive metabolites secreted from Es1. They included ethyl acetate, acetone, and chloroform, and a mixture of ethyl acetate and chloroform (1:1; v/v). The results revealed that the best solvent was ethyl acetate. Ethyl acetate was reported as the best solvent used for extraction of active metabolites from *Penicillium* species (Petit *et al*., 2009). In contrast Svahn *et al*. (2015) used methanol to extract active fractions of bioactive compounds from the solid medium cultures of *Penicillium nalgiovense Laxa* and was fractionated with HPLC as they described. Choice of solvent depends on the chemical nature of the antibiotics.

Collectively, the 47 active isolates were screened using 6 Gram positive and 5 Gram-negative test pathogens including MRSA, ESBL *E. coli* and a yeast (*Saccharomyces cerevisiae*). The number of isolates showing activity using ethyl acetate extraction was less than those in the initial screening. This suggests that studies on the other

isolates should use different solvents. Three isolates (Es1, M1 11 and La1a) were finally chosen according their spectra of activity. These isolates were further subjected to scale up procedure to obtain sufficient crude antimicrobial materials for further purification and chemical identification using HPLC, NMR, MS and FTIR.

Scale up of isolation of antibiotics production by the isolates Es1, M1 11, and La1a

Extracts from 300 petri dishes were pooled, rotary evaporated and redissolved in ethyl acetate. When filtered through 0.22µm filters for HPLC use, some activity was lost after filtration the crude extracts through 0.22µm filters, this loss was probably due to part of antimicrobial compounds being caught in the filter as the compounds were not dissolved totally in the solvent.
Reasonable yields were obtained as crude bioactive materials from Es1 200 ml 7.8 mg/mL (1.650 g from 7.5 L medium), M1 11 200 ml 6.0 mg/mL (1.2g from 7.5 L medium) and from La1a 200 ml 4.6 mg/mL (0.920 g from 7.5 L medium).
A quantitative comparison of the efficacy of the active compounds produced by the three organisms was carried out using paper disc assay method on *E. coli* and *S. aureus*. The results indicated that the active extracted components from Es1 and M1 11 were more active than La1a against *E. coli* and *S. aureus* and Es1 and M1 11 extracts were chosen for further study.

The limitation of this part of the study was that the antibiotics were extracted from just three isolates (Es1, M1 11 and La1a) with broad spectrum activity but in term of commercial potential those producing narrow spectrum antibiotics maybe more applicable to the clinical situation. Also it was not possible to produce a large liquid scale culture which would facilitate production of the crude extract and make the fractionation easier as there would probably be fewer contaminants when using cell free filtered medium than extraction from solid culture. This limitation on the study would need to be overcome if these study to be taken any further.

Chapter 5

Purification and determination of structure of antibiotics

5.1 Introduction

5.1.1 Purification - application of High performance liquid chromatography HPLC

High performance liquid chromatography (HPLC) is a chromatographic technique that is used to separate a mixture of compounds and is applied in biochemistry and analytical chemistry to identify, quantify and purify the individual components of the mixture (Malviya *et al*., 2010). In the early 1970's several reliable separation techniques such as paper chromatography, thin-layer chromatography and open-column chromatography were commonly used in the laboratory. High performance liquid chromatography emerged in the mid 1970's and was widely used in industry as a reliable and efficient separation technique. The technique further progressed with the development of column packing materials and the development of on-line detectors.

In the late 1970's, new methods such as the reverse phase liquid chromatography allowed for further development in separation between compounds with similar properties (Lee, 2009). New techniques were introduced to develop the separation, identification, purification and quantification of HPLC with the addition of computers and automation. The developments of the different types of columns including micro-column and affinity columns have further lead to the improvement of the reproducibility and reliability and eventually fast HPLC made an appearance (Sailaja *et al*., 2014). The HPLC is made up of multiple parts including a computer, mobile phase reservoirs, pump, injector, column, detector and fraction collector (Figure 5.1). The most vital part of the instrument is the column where the separation happens. The components are separated when the analytes and mobile phase are pumped through the column (Kupiec, 2004).

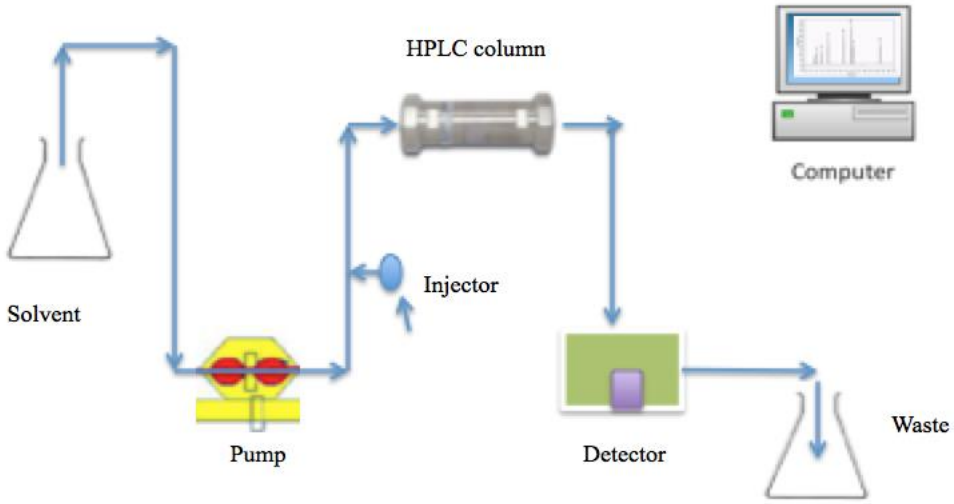


Figure 5.1: The HPLC system and its components, modified from (Bhardwaj *et al*., 2015b)

The types of HPLC are commonly dependent upon the phase system used in the method, these include; normal phase HPLC (NP- HPLC), this approach uses a polar stationary phase and a non-polar mobile phase. The reversed phase HPLC (RP-HPLC) is the most preferred technique for the analytical and preparative separations of desired compound in chemical, biological, pharmaceutical, food and biomedical sciences. In this technique the stationary phase is made up of non-polar hydrophobic molecules with a functional group octyl or octa-decyl attached to the silica gel, the mobile phase is a polar solvent, the polar compounds get eluted first in this mode whereas the non-polar compounds are retained for longer periods; because the majority of the drugs and pharmaceuticals are naturally polar, it means they are not retained for longer periods and thus elute quicker (Husain *et al*., 2011). Size exclusion chromatography (SEC), also known as gel permeation chromatography is commonly used to separate particles based on their size, this is a powerful method for protein purification and polymer characterization (Khodabandehloo *et al*., 2017).
In ion-exchange chromatography, retention is dependent upon the interactions between solute ions and charged sites that are bound to the stationary phase, similarly charged ions are excluded, this technique is more appropriate for separating molecules with a charge only such as proteins, enzymes, peptides, amino acids by itself or in combination with other chromatographic techniques (Acikara, 2013). Bio-affinity chromatography is a technique based on particular reversible interaction of

proteins with ligands, due to the specificity of interaction can lead to very high purification in a single step, this technique is preferably used to isolate proteins and enzymes from complex mixtures (Verma, 2014, Malviya *et al*., 2010, Sabir *et al*., 2013).

Among all the different modes, the reversed-phase chromatography is the most widely used because of its extensive application range, particularly when separating natural products from plant materials. This mode is popular for its simplicity, versatility and range as it can be used with compounds in a broad range of polarity and molecular mass. The separation device of reverse phase chromatography is reliant upon the hydrophobic binding interaction between the solute of the mobile phase and the immobilized hydrophobic material of the stationary phase (Prathapa *et al*., 2013)

5.1.2 Characterisation

5.1.2.1 Nuclear Magnetic Resonance Spectrometry (NMR)

NMR spectroscopy is an analytical technique that involves the application of a magnetic field to a chemical compound and measurement of the spin state of nuclei in the molecule. It gives information on the types of atoms in the compound, the relative amounts of different atoms and information about the structure of the compound. Over the years the use of nuclear magnetic resonance (NMR) has grown exponentially as a spectroscopic instrument and its applications. Now, the use of NMR is applied over a wide range of scientific disciplines including biology, physics and medicine. Due to greatly increased resolution in NMR spectrometers, NMR spectra have become a vital instrument in the study of newly synthesized as well as natural products derived from plants and microorganisms. Since the invention of superconducting magnets together with recently developed, highly complex pulse methods have provided chemists with the ability to define 3-dimentional structures of large molecules in solutions. NMR techniques are increasingly used as a quantitative analysis for multiple purposes including: to analyze impurities in drugs; to distinguish the composition of drug products; and to study metabolites of drugs within body fluids (Holzgrabe *et al*., 1998).

NMR spectroscopy has become a vital instrument to chemists and is used regularly to analyze the chemical structure of basic molecules using 1-dimensional methods (1D-NMR), whereas 2-dimensional methods (2D-NMR) are used to define the structure of

more complex molecules (Webb, 2009). The nuclei of multiple elemental isotopes have a particular spin with the most common isotopes used in organic chemistry being C and H. The NMR behaviour with regards to the nuclei of these two particular elements has been utilized by organic chemist as they yield crucial information that can be used to determine the structure of organic compounds. The analysis of 1D proton spectra (^{1}H NMR) e.g. in the study of plant products is complicated due to majority of the signals overlapping. However the establishment of additional spectral dimensions has made it possible to simplify these spectra and acquire more information, thus the development of 2D NMR was a crucial step in the advancement of NMR spectroscopy. There are multiple forms of 2D NMR experiments; these include Correlation Spectroscopy (COSY, correlation between protons that are coupled to each other), Heteronuclear Multiple Quantum Correlation Spectroscopy (HMQC, used to correlate proton and carbon signals using either one bond or longer range couplings) and Heteronuclear Multiple-bond Correlation Spectroscopy (HMBC). This technique is similar to HMQC, but it is optimized to detect proton-carbon correlation over 2 and/or 3 bonds (Timothy and Claridge, 2009).
NMR provides valuable information in identifying the molecular structure of particular compounds, for example, the number as well as types of protons in the desired molecule. These protons are represented as a NMR signal and give data as it relates to the proton environment and coupling partners. Regardless, this data requires a precise analysis before settling for a final structure. This can be acquired through multiple automated structure elucidation software, which has been developed to clarify the structure of the interpretation methods (Heftmann, 2004, Queiroz *et al*., 2002)

5.1.2.2 Fourier-transform infrared spectroscopy (FTIR*)*

FTIR is most useful for identifying chemicals that are either organic or inorganic (Grumezescu, 2017). FTIR has been recognized as a crucial instrument for the classification and identification of compounds or functional groups (chemical bonds) existent in an unidentified mixture of compounds (Eberhardt *et al*., 2007). The wavelength of light absorbed is characteristic of the chemical bonds. By interpreting the infrared absorption spectrum, the chemical bonds in a molecule can be determined**.** Additionally, the FITR spectra of pure compounds are typically

distinctive that they are recognized as molecular "fingerprint". For majority of the materials the spectrum of an unknown compounds are recognized when they are compared to a library of known compounds (Upadhyay, 2011, Sasidharan *et al*., 2011). To identify less common materials, IR will need to be combined with NMR, mass spectrometry (MS), and/or other techniques (Upadhyay, 2011).

5.1.2.3 Mass spectroscopy

Mass spectrometry (MS) contributes a vital role in the research work relating to phytochemical and small molecules. MS typically provides data as it relates to molecular mass/formula and fragmentation patterns. MS is commonly considered as the most sensitive and precise multipurpose analytical technique and it is an impressive analytical technique with multiple functions such as to quantify known materials, to identify unknown compounds, and discovering molecular structures (Silberring, 2002).

Mass spectrometers are used in a broad range of applications in multiple discipline including chemical, electronics, food processing, petroleum, and pharmaceutical industries. Mass spectrometers examine materials based on their mass-to-charge ratio (m/z) of constituent molecules. To accomplish this, chemical compounds are initially ionized, then separated based on m/z ratio, and lastly detection to produce a significant output for the user (Figure 5.2). The separation and ionization processes occur in a vacuum. Ideally, samples are introduced into the vacuum system of the MS instrument; the neutral molecules are ionized and then carried on towards the mass analyzer. The analyzer runs on a low pressure ($\sim 10^{-4}$–10^{-8} Torr) whereby the ions are separated based on their mass-to-charge ratio, using electric or magnetic fields or a combination of both. The separated ions are then detected and generated signals are processed and displayed on a screen as a mass spectrum. A mass spectrum is a plot of relative abundance (signal intensity) against m/z ratio. The most common ionization techniques used with molecular MS are Electron impact ionization, Chemical ionization, Desorption ionization, Electrospray ionization and Ambient ionization (Maher *et al*., 2015).

Electron impact ionization (EI), in the early stages of mass spectrometry EI was the oldest of ionization methods used and it was the first molecular ionization method used create gas-phase ions for a wide range of organic molecules. This method can be applied to compounds which are easily vaporized. EI generates positive ions due to

electron ejection from the sample (Maher *et al*., 2015)

Chemical ionization (CI), this method uses gas-phase chemical reactions to ionize molecules via charge exchange or proton transfer with little or no fragmentation compared to EI so it is considered a soft ionization methods (Munson and Field, 1966). Both of methods mentioned above (EI and CI), the sample molecule is originally in the gas phase. These gas-phase sample molecules created by the volatilization process must not decomposition or reorganization the sample.

Due to the limitation of EI and CI just for volatile compounds this limitation led to the evolution of desorption ionization methods to create ions from non-volatile sample molecules and more delicate biological molecules (Busch, 1995).

Desorption ionization (DI) such as photoionization. Photoionization is independent of surrounding molecules and involves photon absorption followed by ejection of an electron. Accordingly probing of state-selected molecular fragmentation dynamics can be achieved by coincidence measurements of several particles (Miron and Morin, 2009). This has led to ultrafast molecular dissociation mechanisms being proposed (Travnikova *et al*., 2013).

Electrospray ionization (ESI), the molecular- weight range of MS increased by this method, making it achievable to ionize a wide range of compounds including large molecular-weight bio- molecules that had before been difficult to analyze (Heck and Van den Heuvel, 2004).

Ambient ionization method, this method has attracted much attention in many fields as pharmaceuticals, food safety and biomedicine, in this method requires minimal or no sample pretreatment. It usually involves separate ionization and sample-introduction events, allowing independent control over each set of conditions. Ionization is typically achieved under ambient environments through use of existing electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) techniques. Rabid analyses possible to the gas, liquid, and solid samples with the adoption of various sample-introduction methods (Huang *et al*., 2010).

The aims of this part of the study were to fractionate and purify the active compounds from crude extracts and to determine their chemical structure.

Table 5.1: Ionization methods commonly used for molecular MS.

Method	Agent(s)	Sample phase	Hard or soft
Electron impact ionization	Energetic electrons	Gas	Hard
Chemical ionization	Reagent gas-phase ions	Gas	Soft
Desorption ionization (e.g., fast atom bombardment, photoionization, plasma desorption)	Energetic ions, photons, plasma	Solid, liquid, gas	Soft
Electrospray ionization	Highly energetic charged droplets	Liquid	Soft
Ambient ionization (e.g., direct analysis in real time, desorption electrospray ionization)	Highly energetic charged droplets, photons, plasma	Solid, liquid, gas	Soft

"Soft" ionization means to the formation of a large proportion of ions without breaking chemical bonds. "Hard" ionization results in chemical bonds being broken and formation of a large proportion of fragment ions.
Modified from Maher *et al*. (2015).

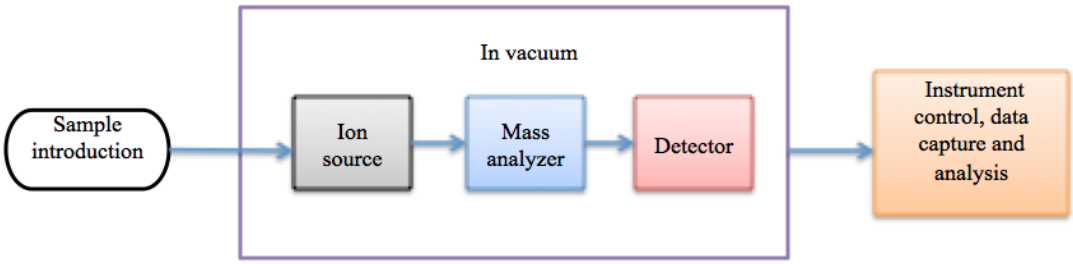


Figure 5.2: Mass spectroscopy instrument, modified from (Maher *et al*., 2015)

5.2 Results

5.2.1 Optimization of fractionation by HPLC

The separations were conducted by injecting 20 µl of the extract (from isolates Es1 and M1 11) onto the analytical column. Six solvent systems were used for optimisation of the analytical RP-HPLC parameters using isocratic elution with different mobile phases as described in section 2.17.1.

Es1 extract

The resultant chromatograms are shown in Figures 5.3 to 5.7. The result showed that the crude extract could be bound to the column and not eluted (an isocratic mobile phase of water: methanol 70:30 (v/v) Figure 5.3, an isocratic mobile phase of water: methanol 50:50 (v/v) Figure 5.4 and an isocratic mobile phase of water: methanol 40:60 (v/v) Figure 5.5) or could be resolved into 5 components with RT of 1.055, 2.971, 3.157, 3.291 and 5.620, arrows in the Figure 5.7 (an isocratic mobile phase of water: methanol 10:90 (v/v) Figure 5.7) to 19 components (an isocratic mobile phase water: methanol 30:70 (v/v) Figure 5.6). The 30:70 (v/v) of water: methanol isocratic system gave a better separation than the other mobile phases, their was no further improvement by adding TFA in separation see Appendix 2.1

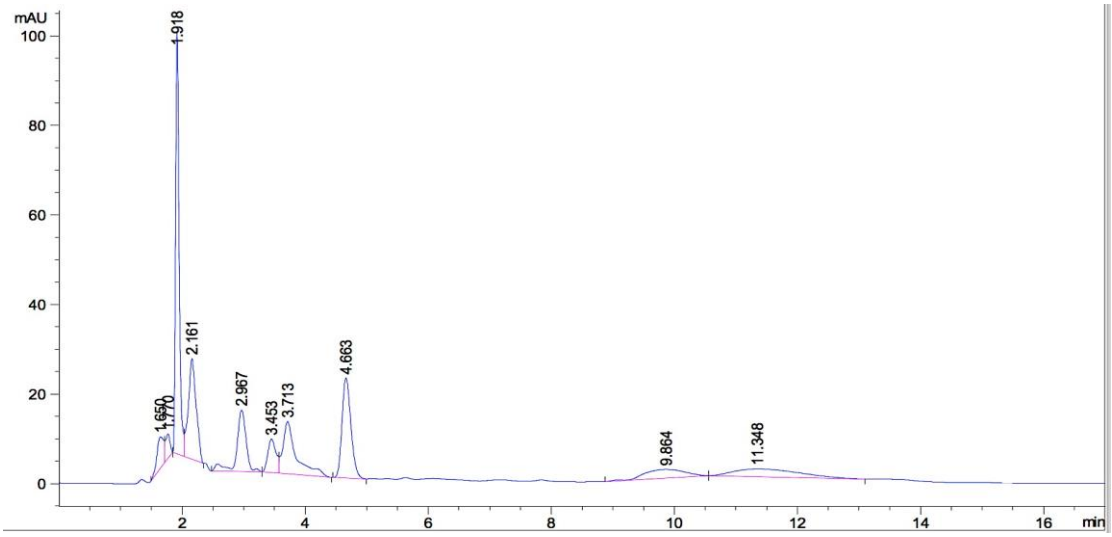


Figure 5.3: Fractionation of Es1 extracts by HPLC on Spherisorb, using a mobile phase of water: methanol 70:30 (v/v)

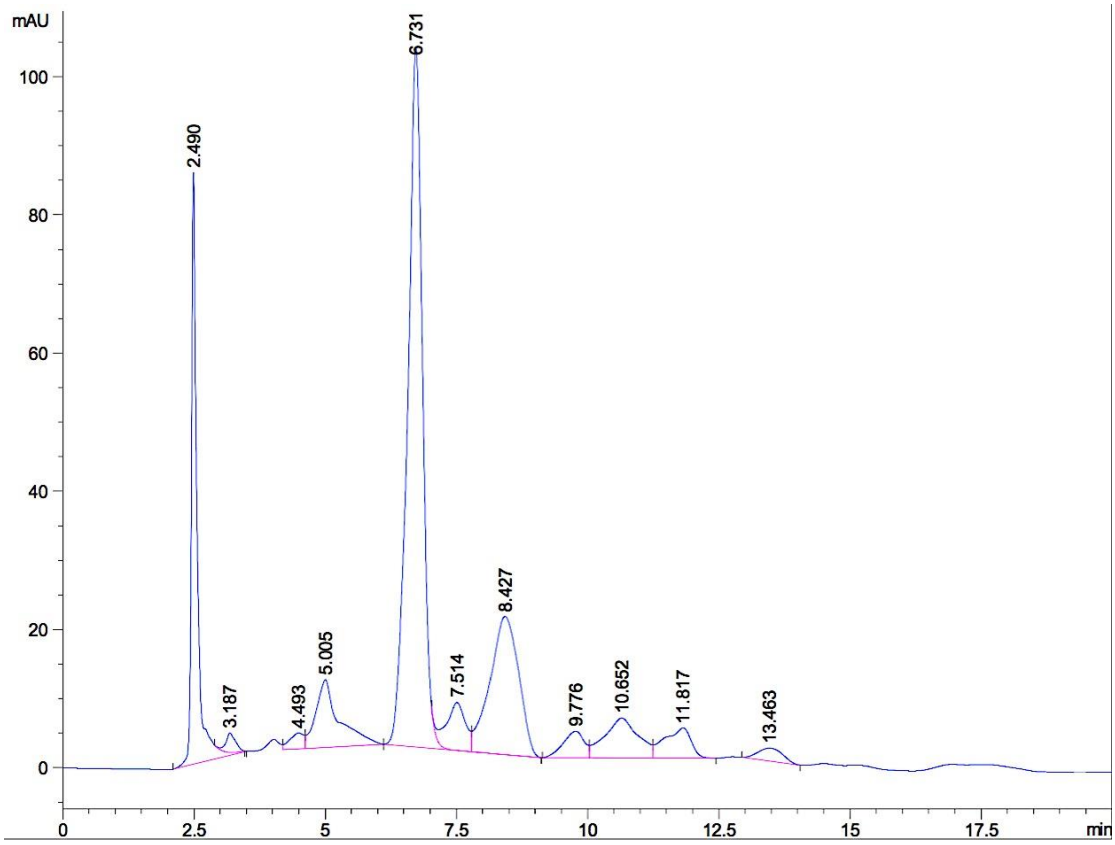


Figure 5.4: Fractionation of Es1 extracts by HPLC on Spherisorb, using an isocratic mobile phase of water: methanol 50:50 (v/v)

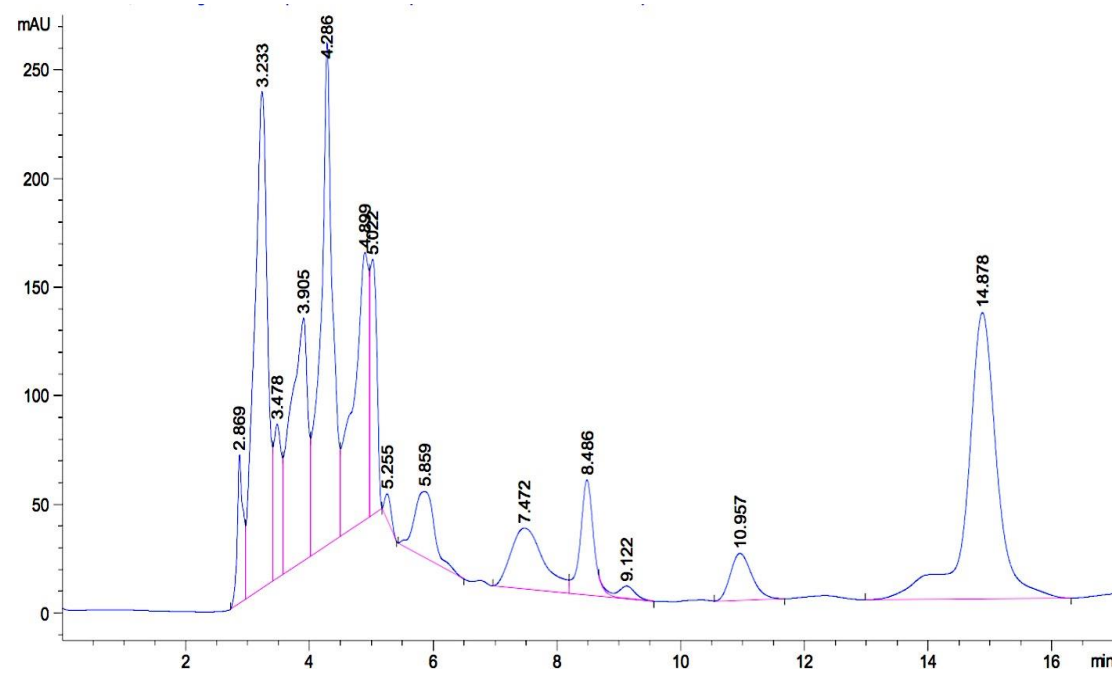


Figure 5.5: Fractionation of Es1 extracts by HPLC on Spherisorb using an isocratic mobile phase of water: methanol 40:60 (v/v)

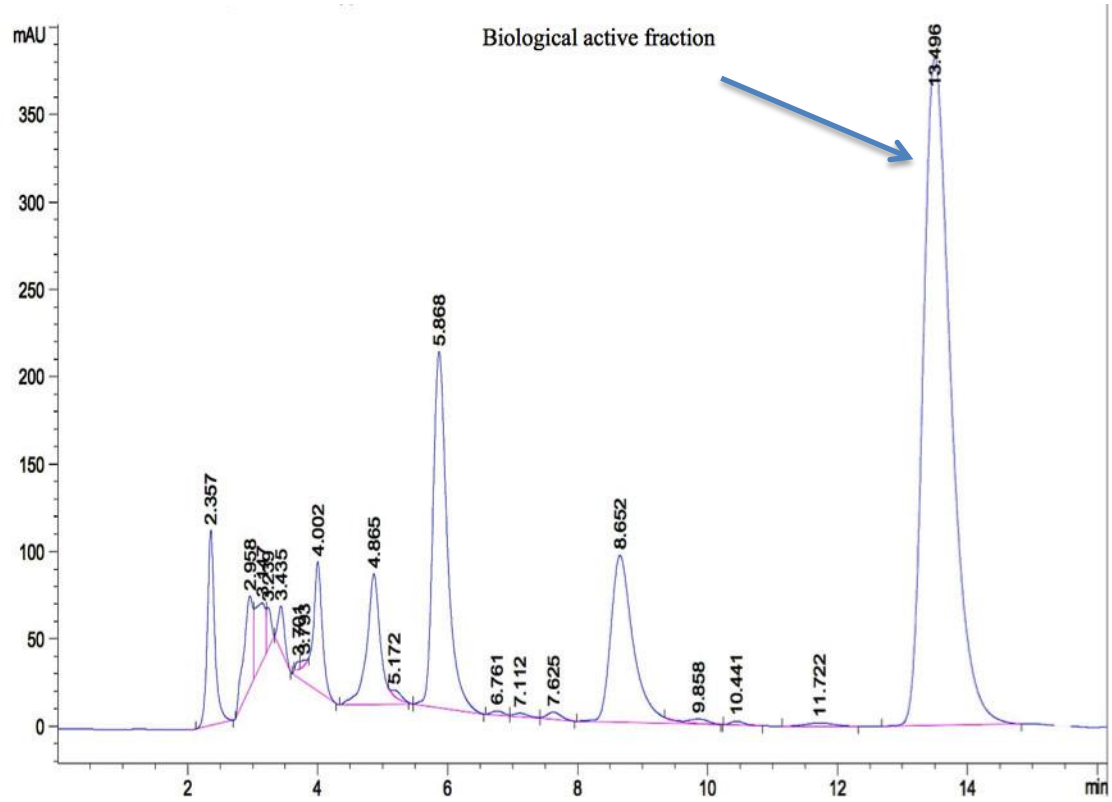


Figure 5.6: Fractionation of Es1 extracts by HPLC on Spherisorb using an isocratic mobile phase water: methanol 30:70 (v/v)

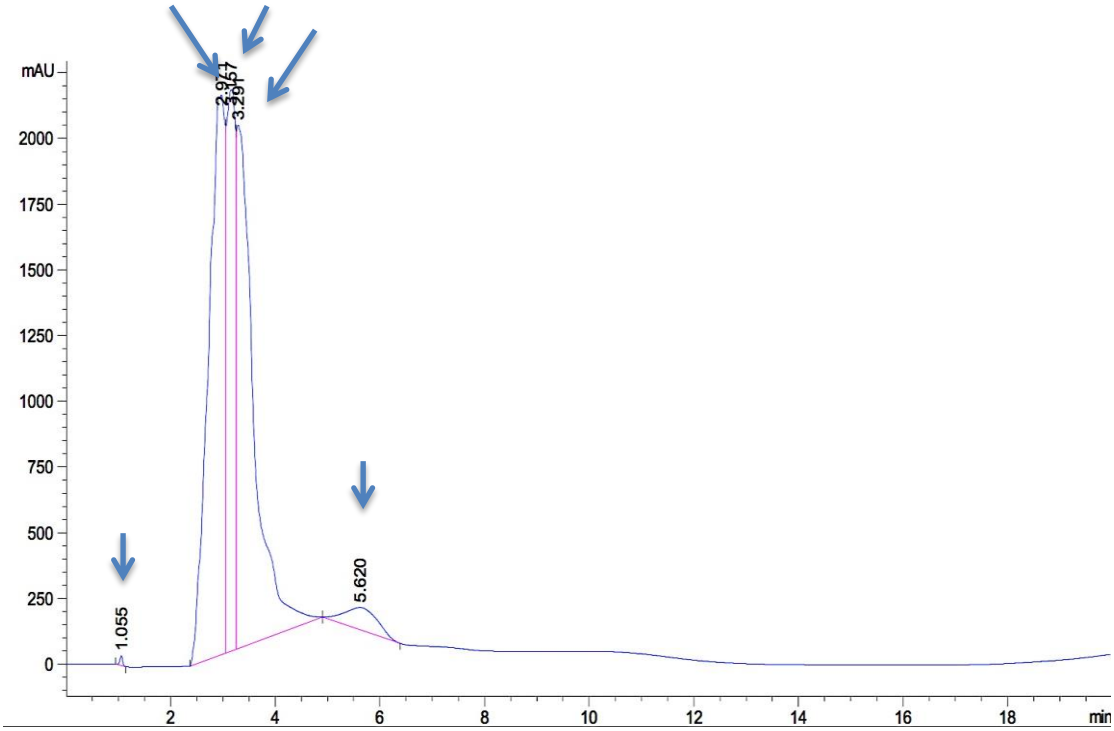


Figure 5.7: Fractionation of Es1 extracts by HPLC on Spherisorb, using an isocratic mobile phase of water: methanol 10:90 (v/v)

The different scales used on the y axes is due to differences in the elution and fractionation of the material, Figure 5.7 the crude extract eluted, fractionated and

resolved into five peaks (fractionation of the crude extract was not successful), Figure 5.6 the crude extract eluted, fractionated and resolved into 19 peaks (better fractionation).

The fractions from the better separation mobile phases (water: methanol 30:70 (v/v)) were tested for antibacterial activity and one fraction (with a retention time 13.496 min: Figure 5.6) gave a large inhibition zone on plates seeded with *E.coli* and *S. aureus.*

M1 11 extracts

The resultant chromatograms are shown in Figures 5.8 to 5.11. The 30:70 (v/v) of water: methanol isocratic system gave a better separation than the other mobile phases. The results showed that the crude extract was not well resolved (Figures 5.8 to 5.10) but with an isocratic mobile phase water: methanol 30:70 (v/v) resolved into 15 components (Figure 5.11).

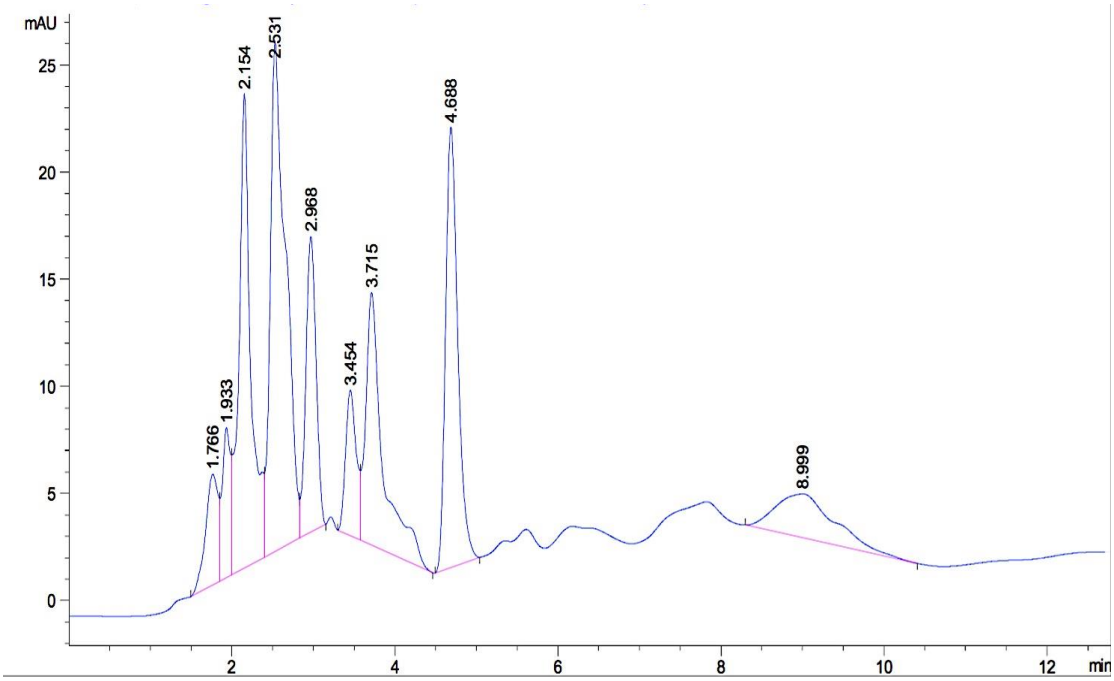


Figure 5.8: Fractionation of M1-11 extracts by HPLC on Spherisorb, using an isocratic mobile phase of water: methanol 70:30 (v/v).

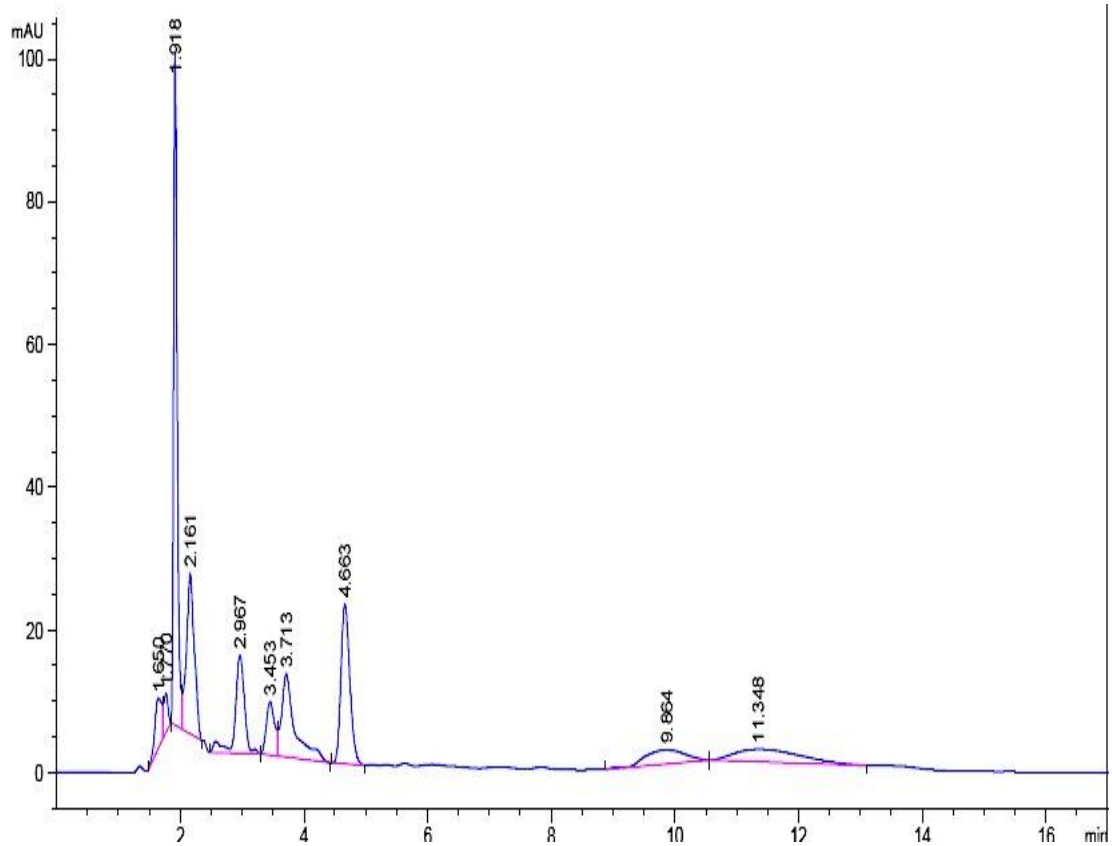


Figure 5.9: Fractionation of M1-11 extracts by HPLC on Spherisorb, using an isocratic mobile phase of water: methanol 50:50 (v/v).

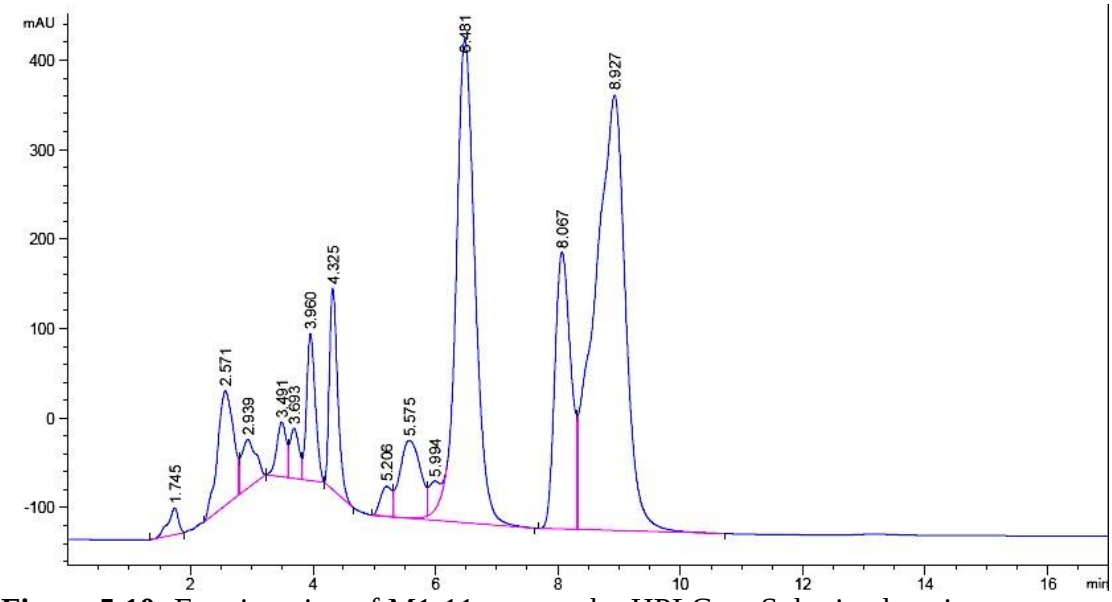


Figure 5.10: Fractionation of M1-11 extracts by HPLC on Spherisorb, using an isocratic mobile phase of water: methanol 40:60 (v/v)

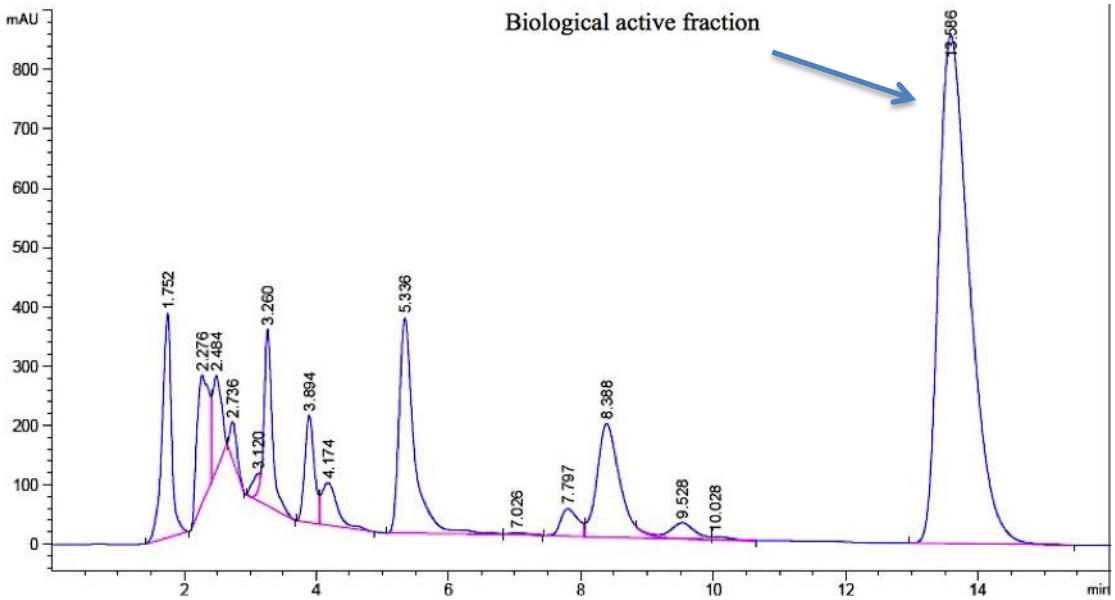


Figure 5.11: Fractionation of M1-11 extracts by HPLC on Spherisorb, using an isocratic mobile phase of water: methanol 30:70 (v/v).

The fractions of the 30:70 (v/v) of water: methanol isocratic system were collected and tested for antibacterial activity, one fraction with a retention time of 13.586 min (Figure 5.11) gave large inhibition zones on plates seeded with *E.coli* and *S. aureus.*

Purification of the antibiotics from Es1 extracts

Use of different HPLC columns for purification of Es1 extracts

Three analytical columns, ACE Excel 5 C-18 amide, ACE Excel 5 SuperC18 and ACE Excel 5 CN-ES were tried using isocratic elution with a mobile phase of water: methanol 30:70 (v/v), flow rate 1 ml/min. Twenty microlitres of Es1 extract was injected the onto each column. The chromatograms are shown in Figures 5.12 to 5.14. The column that gave the best separation was ACE Excel 5 C-18 amide column. The fractions of ACE Excel 5 C-18 amide column were collected separately and tested for antibacterial activity. Three fractions with retention times of 12.230 (F3), 27.272 (F5) and 46.390 (F6) min (Figure 5.14) gave inhibition zones around 6 mm filter paper disks on plate seeded with *S. aureus* ATCC 25923 (F3 4mm, F5 5mm and F6 11mm), rest of the fractions showed no inhibition zones (Figure 5.15).

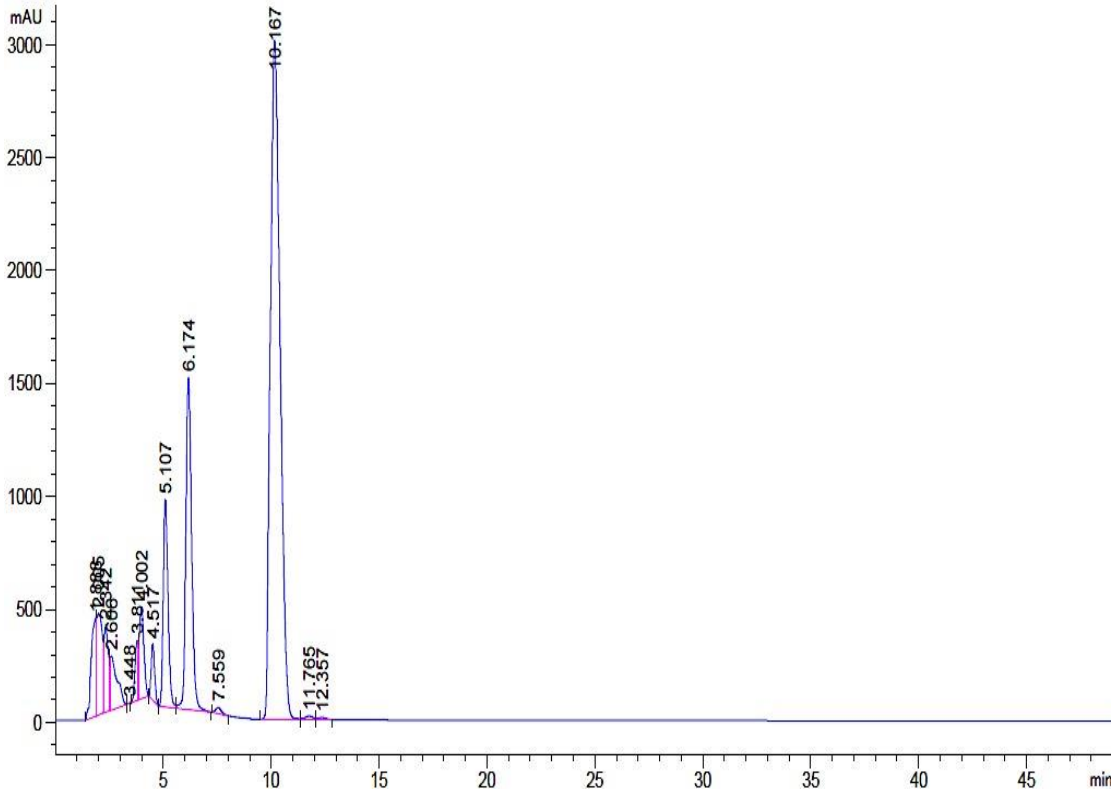


Figure 5.12: Fractionation of ES1 extract by HPLC, 20 µl Es1 extract was injected into ACE Excel 5 SuperC18, the solvent system was isocratic mobile phase water: methanol 30:70 (v/v).

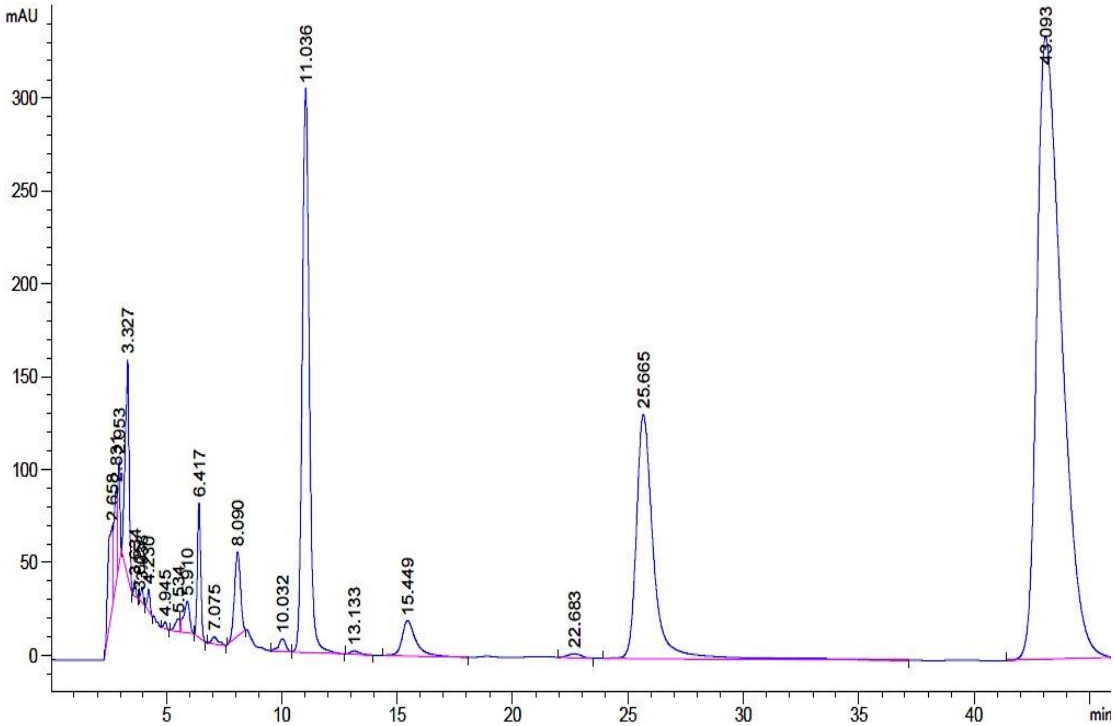


Figure 5.13: Fractionation of ES1 extract by HPLC, 20 µl Es1 extract was injected into ACE Excel 5 CN-ES, the solvent systems was isocratic mobile phase water: methanol 30:70 (v/v).

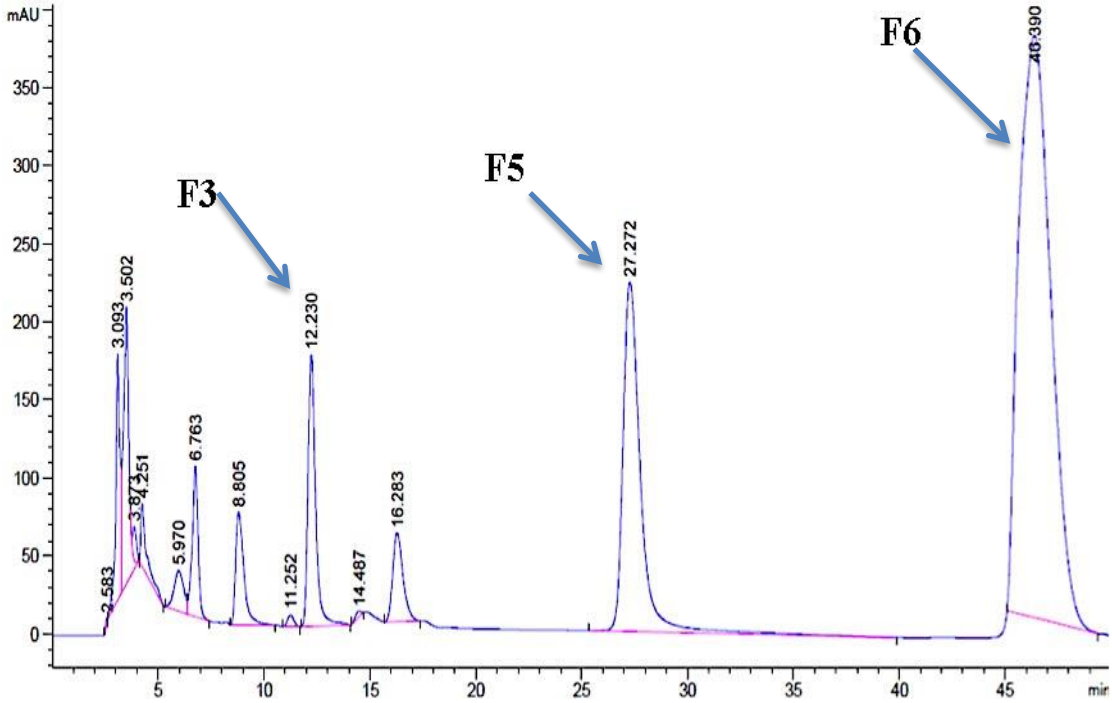


Figure 5.14: Fractionation of ES1 extract by HPLC, 20 µl Es1 extract was injected into ACE Excel 5 C-18 amide, the solvent systems was isocratic mobile phase water: methanol 30:70 (v/v)

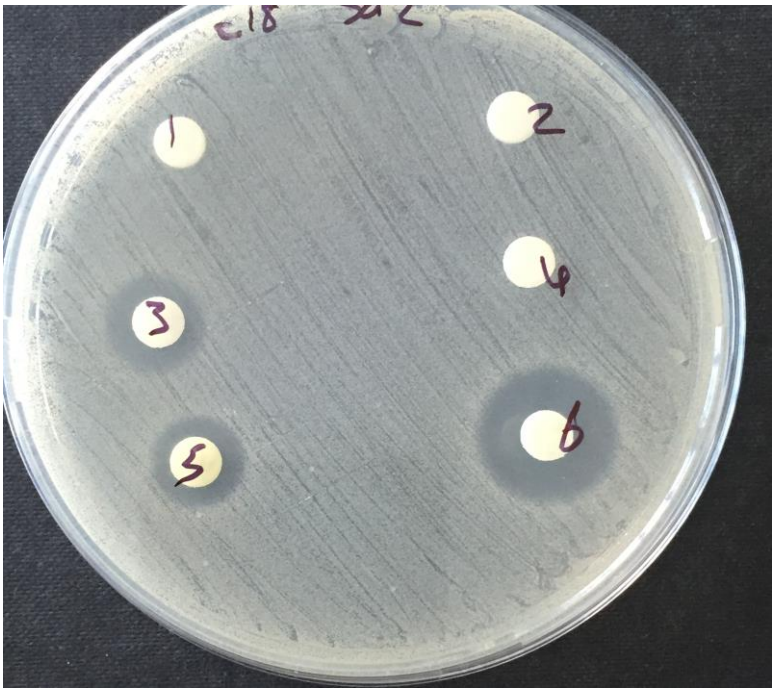


Figure 5.15: MHA plate seeded with *S. aureus* ATCC 25923 showing inhibition zones around 6 mm filter paper disks loaded with fractions collected from Es1 extract separated by ACE Excel 5 C-18 amide, the solvent systems was isocratic mobile phase water: methanol 30:70 (v/v). Three fractions showed inhibition zones (F3 4mm, F5 5mm and F6 11mm)

Comparison of fractions prepared by HPLC of Es1 extracts from solid medium (AIA), broth medium (AIB) and mycelium obtained from cells grown AIB.

Analytical ACE Excel 5 C-18 amide was used with a mobile phase of water: methanol 30:70 (v/v). The chromatogram was conducted by injecting equal amounts of the three extracts 9AIA, AIB and mycelium extracts) and the peaks of the chromatogram were collected separately and tested for antibacterial activity. The results showed that three biological active fractions were present in all extracts with different concentrations, considering the area under the peaks as an indicator of concentration (Tables 5.2 to 5.4).

Three fractions with retention times of 11.382 (F3), 23.182 (F5), 39.122 min (F6) were obtained from AIA extract, three fractions with retention times 11.316 (F3), 23.122 (F5) and 39.415 min (F6) from AIB extract and three fractions with retention times of 11.298 (F3), 23.084 (F5) and 39.245 min (F6) from mycelium extract gave inhibition zones in plates seeded with *E.coli* and *S.aureus* (Figures 5.16 to 5.18). The retention times were very similar suggesting that the same compounds were present in each of the extracts. Quantitatively fraction F3 was lowest in the AIB extract but higher in the AIA and mycelium extracts whereas fraction F6 was highest (>50%) in the AIB extract but lower in the other two extracts. Fraction F5 was of relatively low abundance in all three extracts (Tables 5.2 to 5.4).

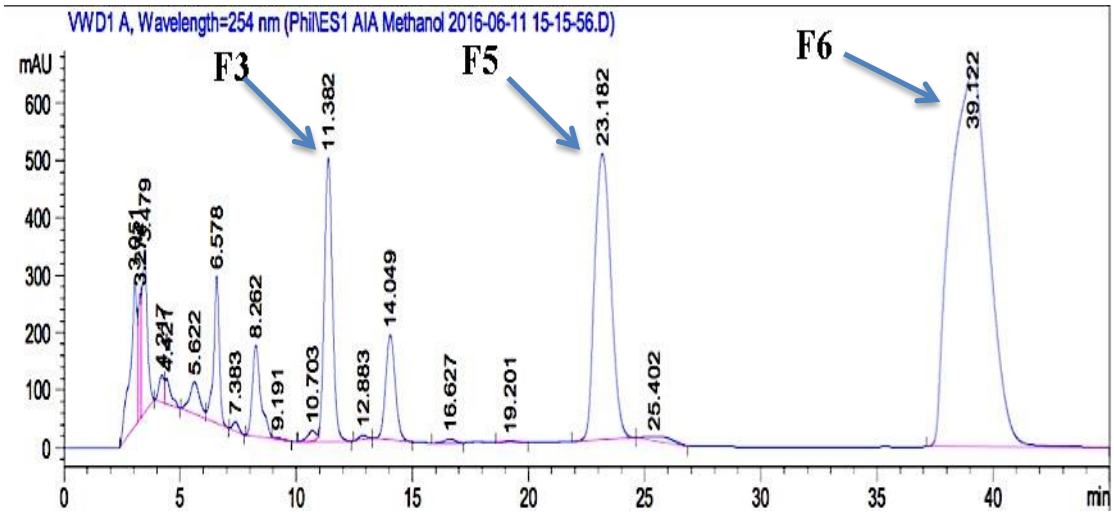


Figure 5.16: Fractionation of injecting 20 µl Es1 extracts from AIA into ACE Excel 5 C-18 amide, flow rate 2ml/min, the solvent systems was isocratic mobile phase water: methanol 30:70 (v/v).

Table 5.2: Showing concentration of the three biological fractions indicated by the area under the peaks (Es1 extracts from AIA)

Signal 2: VWD1 A, Wavelength=254 nm

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %	
1	3.051	BV	0.2544	5064.52881	258.70090	3.5911	
2	3.274	VV	0.1154	1742.58850	217.77338	1.2356	
3	3.479	VB	0.2249	4647.91992	284.09753	3.2957	
4	4.217	BV	0.2041	730.25824	47.83664	0.5178	
5	4.421	VB	0.2432	772.64679	43.71180	0.5479	
6	5.622	BV	0.4441	1696.76965	55.74776	1.2031	
7	6.578	VB	0.2441	4283.20752	256.10495	3.0371	
8	7.383	BB	0.2714	271.21915	15.55697	0.1923	
9	8.262	BV R	0.3744	4044.03149	159.15385	2.8675	
10	9.191	VB E	0.2704	48.46139	2.56473	0.0344	
11	10.703	BV E	0.3552	432.35040	18.46263	0.3066	
12	11.382	VB R	0.3731	1.19330e4	493.49139	8.4614	F3
13	12.883	BB	0.3680	173.22685	7.48423	0.1228	
14	14.049	BB	0.4460	5253.18115	182.94229	3.7249	
15	16.627	BB	0.4946	210.87416	6.62106	0.1495	
16	19.201	BB	0.5801	108.25790	2.86967	0.0768	
17	23.182	BB	0.7844	2.40120e4	498.15472	17.0263	F5
18	25.402	BB	1.1212	685.07269	7.39224	0.4858	
19	39.122	BB	1.5562	7.49191e4	657.10870	53.1233	F6
Totals :				1.41029e5	3215.77547		

Peak labels as: BB indicates a baseline-resolved peak, BV indicates a peak that starts a cluster, VB indicates a peak that ends a cluster, VV indicates a peak within a cluster, E is for estimated peak and R is rounded peak.

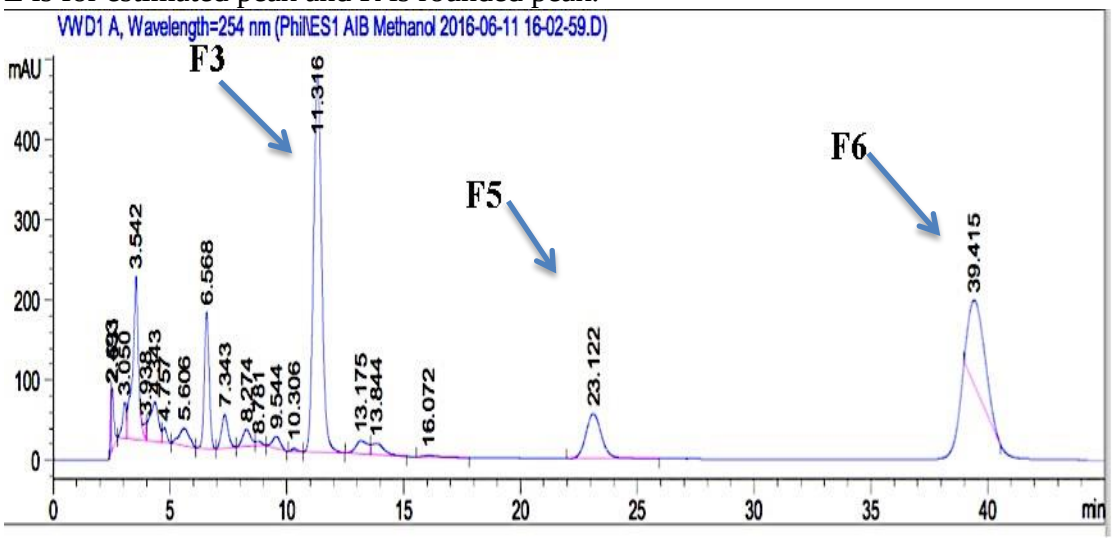


Figure 5.17: Fractionation of injecting 20 µl Es1 extracts from AIB into ACE Excel 5 C-18 amide, flow rate 2 ml/min, the solvent systems was isocratic mobile phase water: methanol 70:30 (v/v)

Table 5.3: Showing concentration of the three biological fractions indicated by the area under the peaks (Es1 extracts from AIB)

Signal 2: VWD1 A, Wavelength=254 nm

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %	
1	2.493	BV	0.0610	292.08304	77.52434	0.9220	
2	2.531	VB	0.0924	494.92545	76.56638	1.5623	
3	3.050	BV	0.1565	499.21210	44.63340	1.5758	
4	3.542	VV R	0.2340	3540.92163	203.53558	11.1775	
5	3.938	VV E	0.1227	153.68680	17.97657	0.4851	
6	4.343	VV	0.3637	1270.19543	48.80086	4.0096	
7	4.757	VB	0.1928	230.14467	17.81005	0.7265	
8	5.606	BB	0.4456	657.52423	21.81734	2.0756	
9	6.568	BB	0.2178	2421.88208	170.45026	7.6451	
10	7.343	BB	0.2859	804.81952	41.93873	2.5405	
11	8.274	BV	0.3703	513.74457	21.23314	1.6217	
12	8.781	VB	0.2710	91.87775	5.25489	0.2900	
13	9.544	BB	0.3560	331.89426	14.55613	1.0477	
14	10.306	BV	0.2400	61.80536	3.94109	0.1951	
15	11.316	VB	0.3733	1.14086e4	471.45135	36.0130	F3
16	13.175	BV	0.5394	600.35223	16.15970	1.8951	
17	13.844	VB	0.5418	509.47708	13.73216	1.6083	
18	16.072	BB	0.6213	84.07180	1.90700	0.2654	
19	23.122	BB	0.7116	2599.60669	56.10456	8.2061	F5
20	39.415	BB	0.7355	5112.18408	104.90929	16.1375	F6
Totals :				3.16790e4	1430.30282		

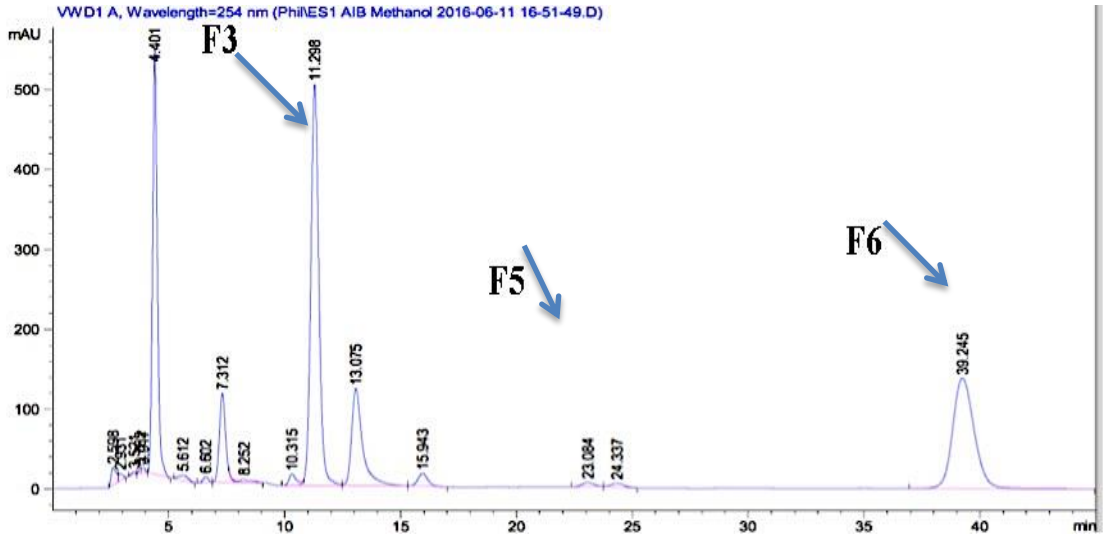


Figure 5.18: Fractionation of injecting 20 µl mycelium extracts from AIB into ACE Excel 5 C-18 amide, flow rate 2 ml/min, the solvent systems was isocratic mobile phase water: methanol 30:70 (v/v).

Table 5.4: Showing concentration of the three biological fractions indicated by the area under the peaks (Es1 extracts from mycelium obtained from cells grown AIB)

Signal 1: VWD1 A, Wavelength=254 nm

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %	
1	2.598	BV	0.2183	329.86478	22.33446	0.8949	
2	2.931	VB	0.1708	104.99738	8.29065	0.2848	
3	3.521	BB	0.1673	39.86955	3.43724	0.1082	
4	3.762	BV	0.1076	39.08921	5.59072	0.1060	
5	3.917	VB	0.1566	66.73765	6.24981	0.1810	
6	4.401	BB	0.2081	7528.47412	531.96204	20.4233	
7	5.612	BB	0.4765	175.94998	6.28634	0.4773	
8	6.602	BB	0.2465	113.04502	7.22675	0.3067	
9	7.312	BV R	0.2845	2101.42749	111.69157	5.7008	
10	8.252	VB E	0.5893	171.83562	3.87572	0.4662	
11	10.315	BV E	0.3159	320.93597	14.68849	0.8706	
12	11.298	VV R	0.3721	1.21852e4	502.19742	33.0560	F3
13	13.075	VB	0.4792	3975.57373	121.68365	10.7850	
14	15.943	BB	0.4550	469.40588	15.82555	1.2734	
15	23.084	BB	0.5564	175.86531	4.97536	0.4771	F5
16	24.337	BB	0.6050	182.85422	4.75149	0.4960	
17	39.245	BB	0.9915	8881.10840	138.97772	24.0927	F6
Totals :				3.68622e4	1510.04499		

Preparation of the fractions with antimicrobial properties from Es1 extracts.

The semi-preparative ACE Excel 5 C-18 amide column was used for collecting the fractions with antimicrobial properties. The results are shown in Figure 5.19

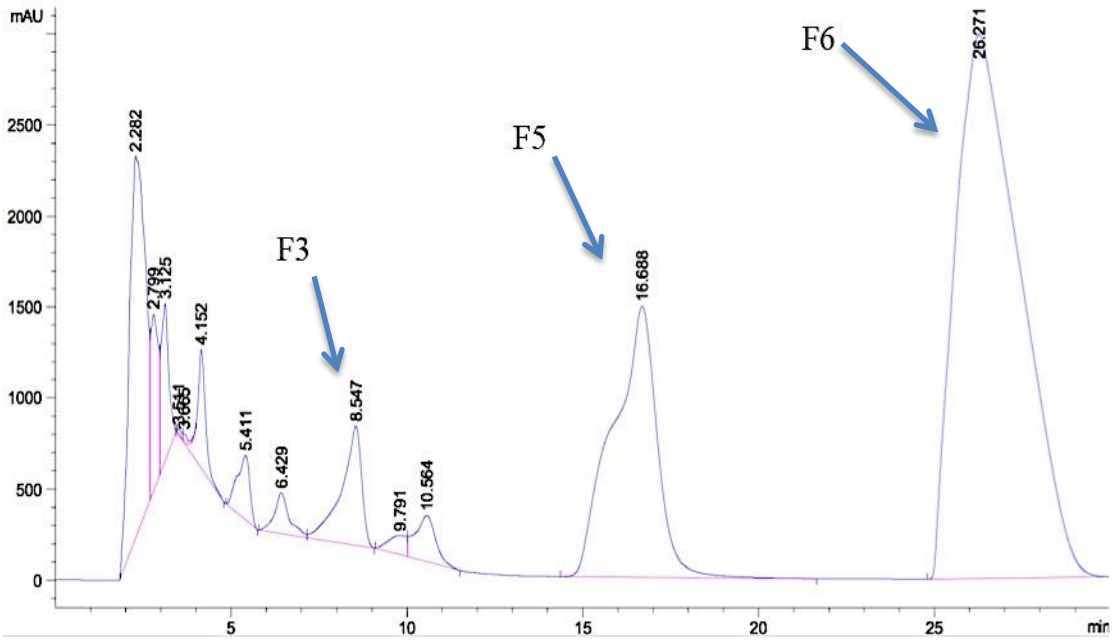


Figure 5.19: Fractions obtained by injecting 500 µl Es1 extracts on to semi-Preparative ACE Excel 5 C-18 amide column, flow rate 5 ml/min, the solvent systems was Gradient mobile phase water: methanol 30:70 (v/v).

Three fractions gave inhibition zone on MHA pates seeded with (*E.coli* and *S. aureus*) at retention times of 8.547 (F3), 16.688 (F5) and 26.271 min (F6).

The whole ES1 extract from 300 plates (200 ml) was injected, 500 µl at a time and the three fractions were collected, pooled, the solvent was removed by rotary evaporation and a brown oil residue obtained, the residue was eluted in methanol-d4, than each fraction analyzed by NMR. More than one compound was present in the proton spectrum in each fraction (data not shown).

Therefore different solvent systems were used to purify the antibiotic from impurities, the best solvent system was isocratic mobile phase water: acetonitrile 33:67 (v/v).

Fraction 3

The results of fractionation of fraction F3 are shown in Figure 5.20. All the peaks were collected separately and the results of antibacterial screening assay indicated that two sub-fractions were active against the test strains and had retention times of 8.405 (F3/1) and 16.139 (F3/3) min.

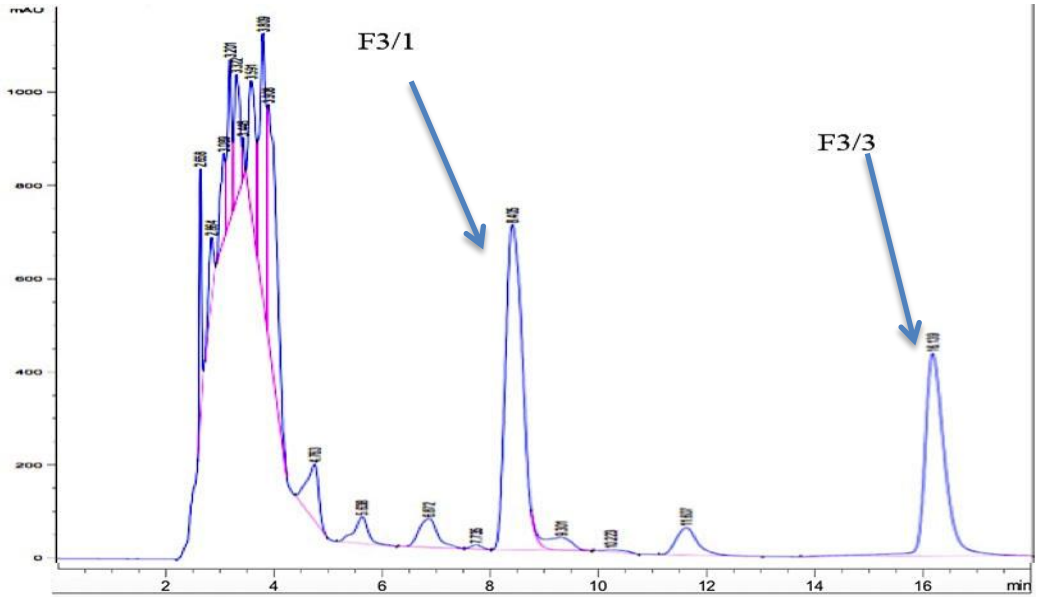


Figure 5.20: Fractions obtained by injecting 250 µl of fraction F3 on to a semi-Preparative ACE Excel 5 C-18 amide column, flow rate 5ml/min, the solvent systems was isocratic mobile phase water: acetonitrile 33:67 (v/v)

Fraction 5

The results of fractionation of fraction F5 are shown in Figure 5.21. There was one major peak with a retention time of 10.159 min and a number of smaller peaks. All low intensity peaks were pooled together and together with the strong peak were and tested for antimicrobial activity. The results showed that the strong peak (F5) demonstrated inhibitory effects against the tested strains.

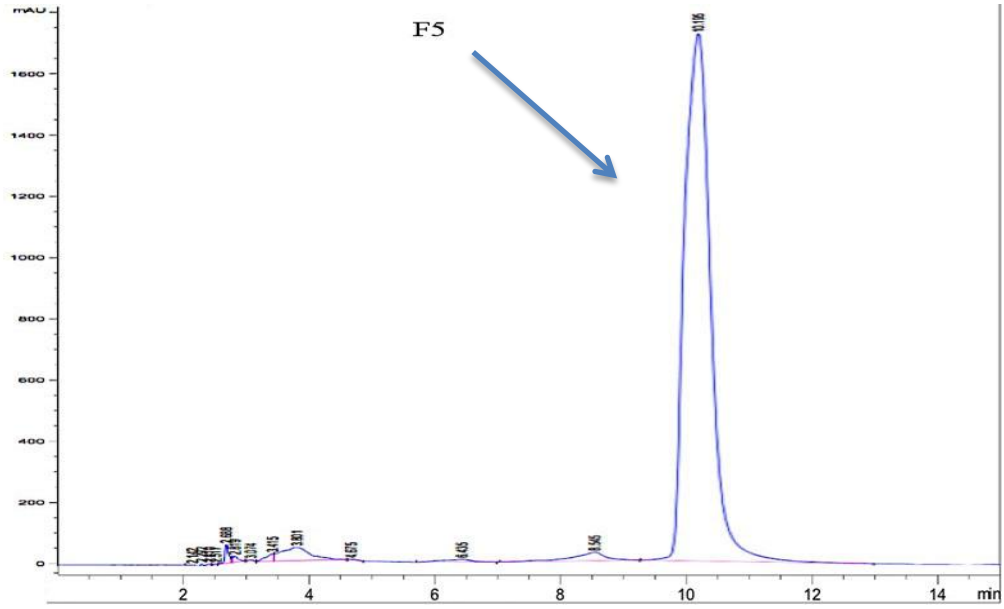


Figure 5.21: Fractions obtained by injecting 250 µl of Fraction F5 on to a semi-Preparative ACE Excel 5 C-18 amide column, flow rate 5 ml/min, the solvent systems was isocratic mobile phase water: acetonitrile 33:67 (v/v).

Fraction 6

The results of fractionation of fraction F6 are shown in Figure 5.22. There were two major peaks with retention times of X and 18.706 min. The results showed that the fraction with a retention time at 18.708 min (F6) had inhibitory effects against the tested strains.

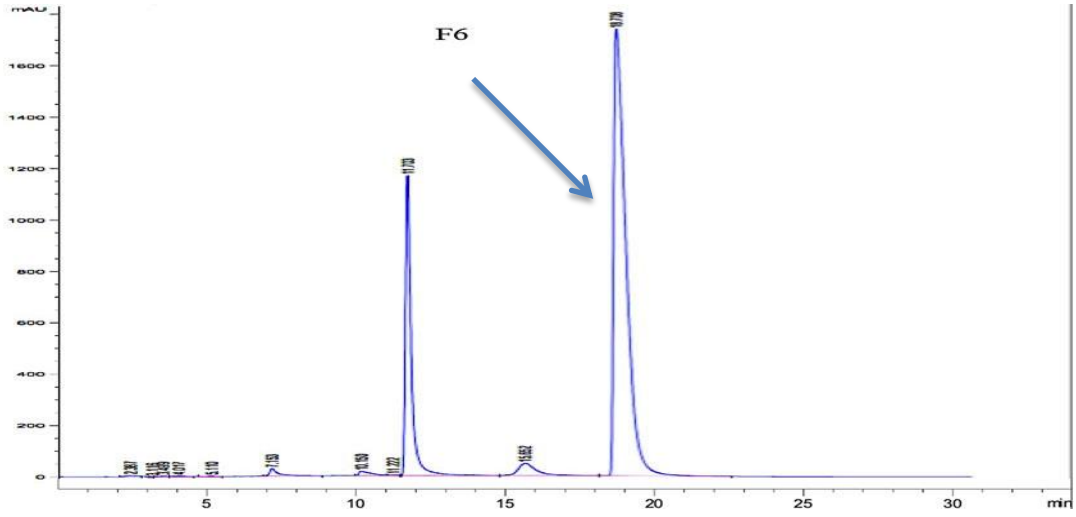


Figure 5.22: Fractions obtained by injecting 250 µl of fraction F6 on to semi-Preparative ACE Excel 5 C-18 amide column, flow rate 5ml/min, the solvent systems was isocratic mobile phase water: acetonitrile 33:67 (v/v).

Fractionation was repeated for all the extracts and the four fractions were pooled and dried by rotory evaporation and weighed. Yields of the purified fractions were F3/1, 3.5 mg; F3/3, 3.7 mg; F5, 10 mg F6, 24 mg. These were then subjected to further analysis by NMR, IR spectroscopy and accurate MS to try to determine the chemical nature and structures of the compounds. The purification procedure is summarised in Table 5.5)

Table 5.5: Summary of purification of the antibiotics for Es1 extraction by HPLC (each step of purification all the fractions collected separately and tested for antimicrobial activity)

Crude extract	First step of the purification, the solvent systems was water: methanol 30:70 (v/v)	Second step of the purification, the solvent systems was water: acetonitrile 33:67 (v/v)
Injecting Es1 crude extract on to reverse phase HPLC	F3 at RT 8.547 min, semi pure fraction	F3/1 (3.5 mg) at RT 8.405 min, pure fraction
		F3/3 (3.7 mg) at RT 16.139 min, pure fraction
	F5 at RT 16.688 min, semi pure fraction	F5 (10 mg) at RT 10.195 min, pure fraction
	F6 at RT 26.271 min, semi pure fraction	F5 (10 mg) at RT 10.195 min, pure fraction
		F6 (24) mg at RT 18.708 min, pure fraction

Purification of the antibiotics for M1 11 extracts

A semi-preparative ACE Excel 5 C-18 amide column with two different solvent system was used. The first solvent system was an isocratic elution using a mobile phase of water: methanol 30:70 (v/v) Figure 5.23, the second was an isocratic elution run using water: acetonitrile with a ratio of 33:67 (v/v) Figure 5.24, both with flow rate 5 ml/min. The second solvent system gave better separation.

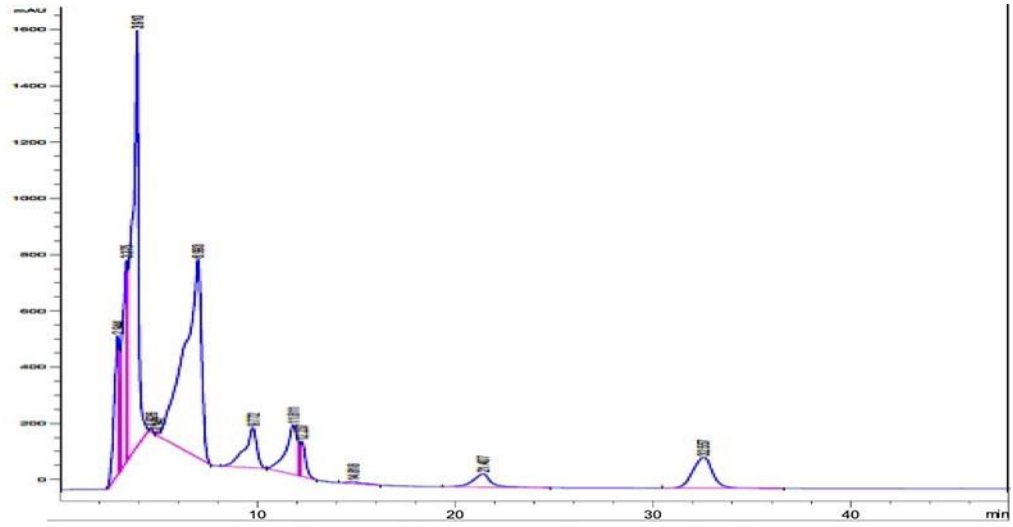


Figure 5.23: Fractions obtained by injecting 250 µl M1 11 extracts on to a semi-Preparative ACE Excel 5 C-18 amide column, flow rate 5ml/min, the solvent systems was isocratic mobile phase water: methanol 30:70 (v/v).

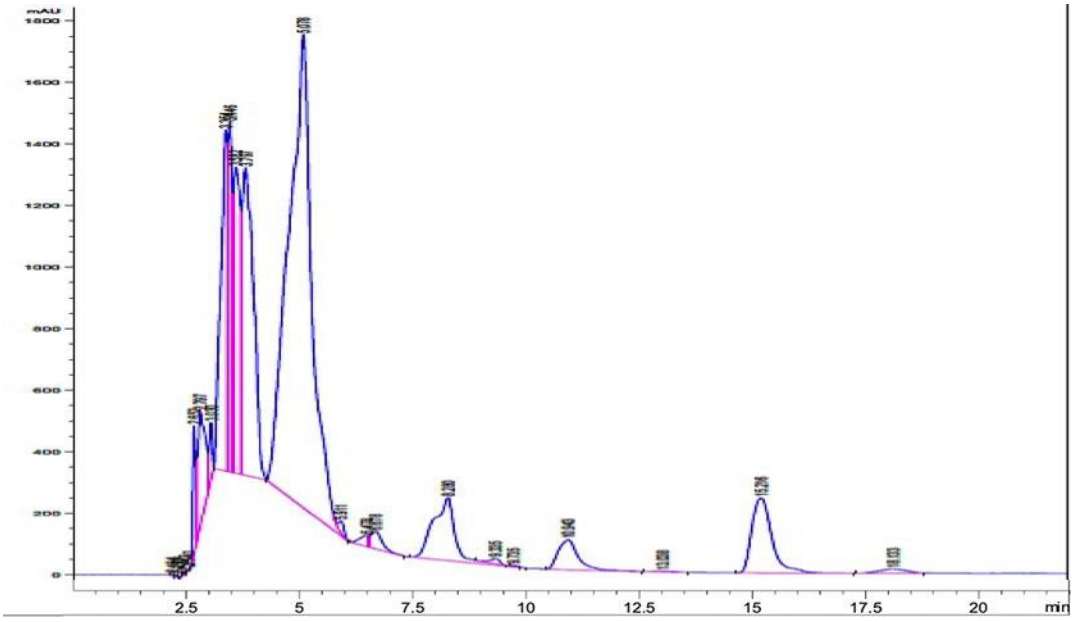


Figure 5.24: Fractions obtained by injecting 250 µl M1 11 extracts on to a semi-Preparative ACE Excel 5 C-18 amide column, flow rate 5ml/min, the solvent systems was isocratic mobile phase water: acetonitrile 33:67 (v/v).

Collecting the fractions with antimicrobial properties from M1 11 extracts.

Solvent system water: acetonitrile 33:67 (v/v) was used to collecting fractions with antibacterial activity.

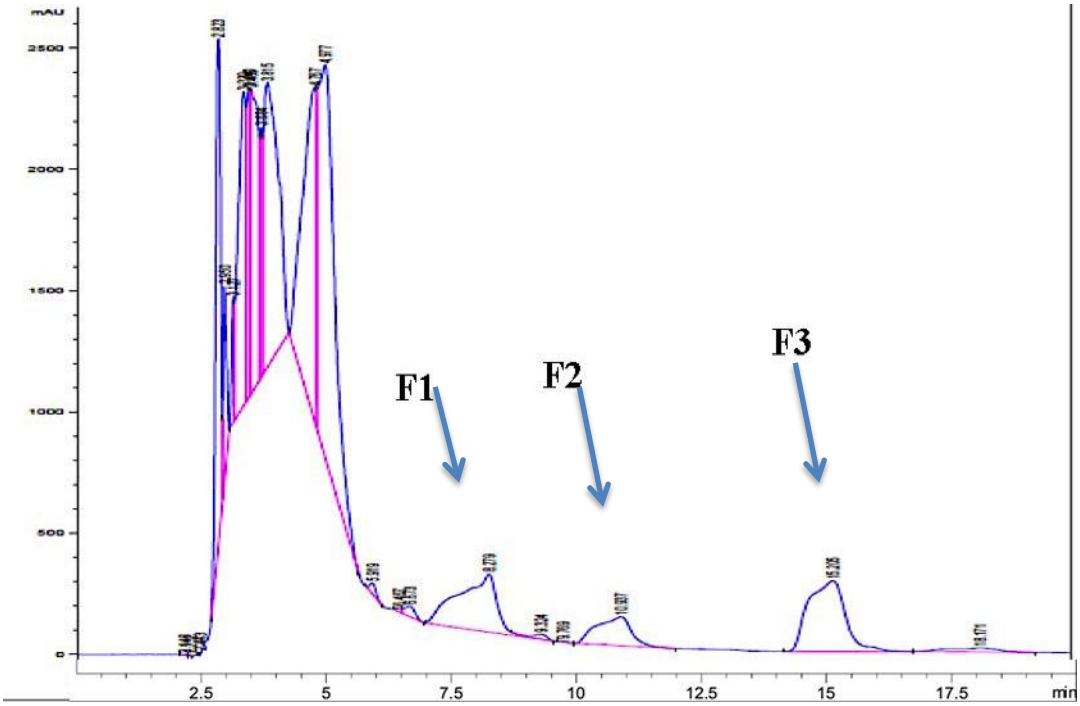


Figure 5.25: Fractions obtained by injecting 500 µl M1 11 extracts on to a semi-Preparative ACE Excel 5 C-18 amide column, flow rate 5 ml/min, the solvent systems was Gradient mobile phase water: acetonitrile 33:67 (v/v).

The fractions were collected separately and tested for antibacterial activity, three fractions gave inhibition zone in seeded plates (*E.coli* and *S. aureus*) at retention times of 8.279 (M1 F1), 10.937 (M1 F2) and 15.205 (M1 F3) mins (Figure 5.25).

All M1 11 extract (200 ml) was injected 500 µl at a time and the three fractions were collected, pooled, solvent removed by rotary evaporator and yellowish brown oil residues obtained. The residues were then dissolved in methanol-d4. Each fraction was analysed by NMR, however the proton spectra showed more than one compound in each fraction.

Different solvent system were used for each fraction to purify the antibiotic from impurities, three solvent systems were used, an isocratic elution using a mobile phase of water: methanol 30:70 (v/v; Figures 5.26, 5.29, 5.32), an isocratic elution run using water: methanol: acetonitrile with a ratio of 35:35:30 (v/v; Figures 5.27, 5.30, 5.33) and an isocratic elution run using water: methanol: acetonitrile with a ratio of

40:40:20 (v/v; Figures 5.28, 5.31, 5.34). The peaks of the chromatogram of the best solvent systems were isocratic mobile phase water: methanol: acetonitrile with a ratio of 30:35:35 (v/v) gave better separation.

Samples (250 µl) of each fraction (M1 F1, M1 F2 and M1 F3) were therefore injected on to a semi-preparative ACE Excel 5 C-18 amide column using an isocratic mobile phase water: methanol: acetonitrile with a ratio of 30:35:35 (v/v). Each peak was collected separately assayed for antimicrobial activity. The results showed that there were four peaks with antibacterial activity from M1 F1, retention time at 4.162 min (F1 1 p3), 7.570 min (F1 2 p4), 9.246 min (F1 3 p5) and 17.529 min (F1 4 p6; Figure 5.27), one peak with antibacterial activity from M1 F2 retention time at 7.660 min (F2 2 p2; Figure 5.30), five peaks with antibacterial activity from M1 F3 retention times at 3.974 min (F3 1 p2), 5.360 min (F3 2 p3), 6.882 min (F3 3 p4), 8.282 min (F3 4 p5) and 14.966 min (F3 5 p6; Figure 5.33).

M1 F1

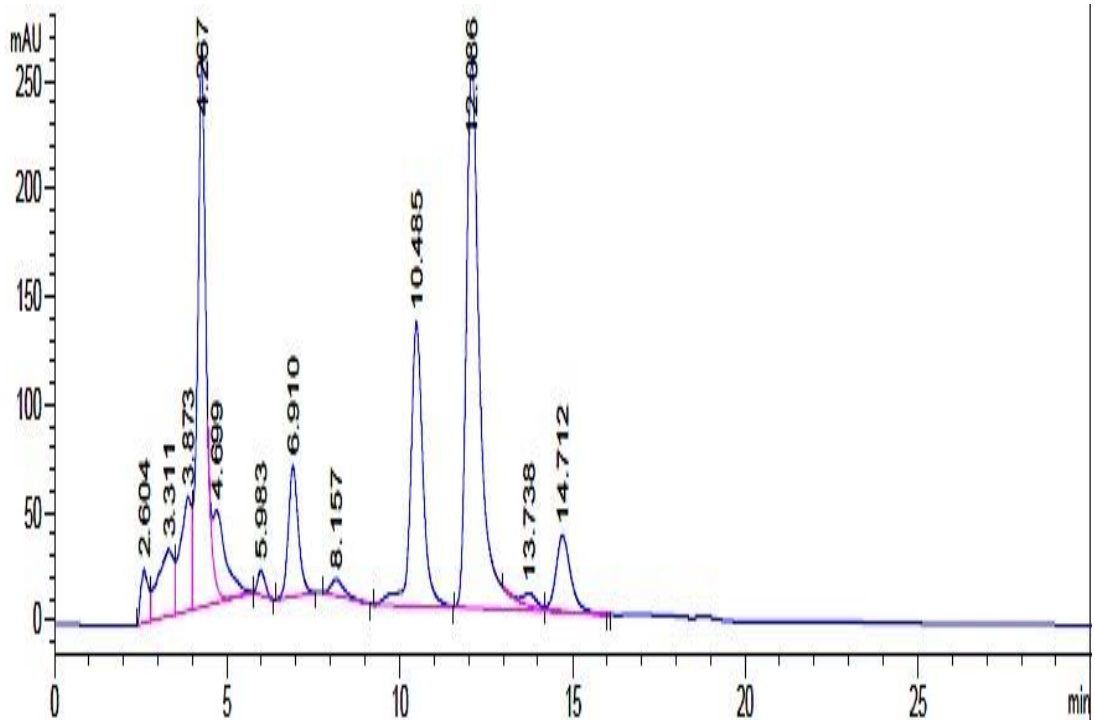


Figure 5.26: Fractions obtained by injecting 250 µl M1 F1 on to a semi-Preparative ACE Excel 5 C-18 amide column, flow rate 5 ml/min, the solvent systems was isocratic mobile phase water: methanol 30:70 (v/v).

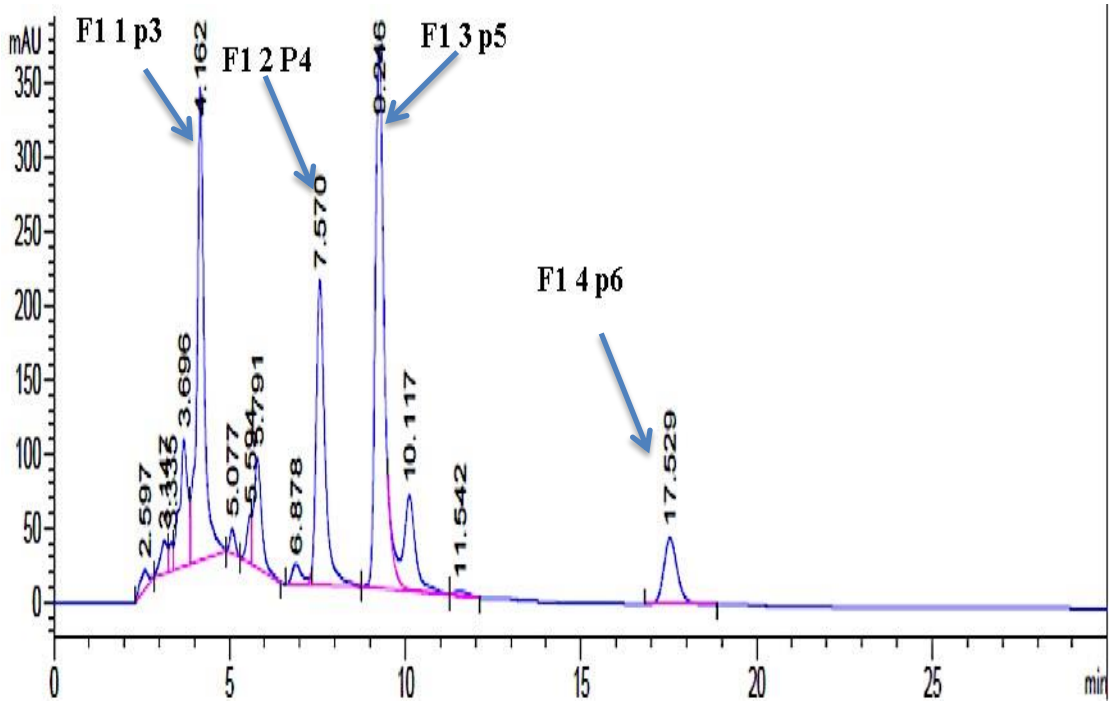


Figure 5.27: Fractions obtained by injecting 250 µl M1 F1 on to a semi-Preparative ACE Excel 5 C-18 amide column, flow rate 5 ml/min, the solvent systems was isocratic mobile phase water: methanol: acetonitrile with a ratio of 30:35:35 (v/v)

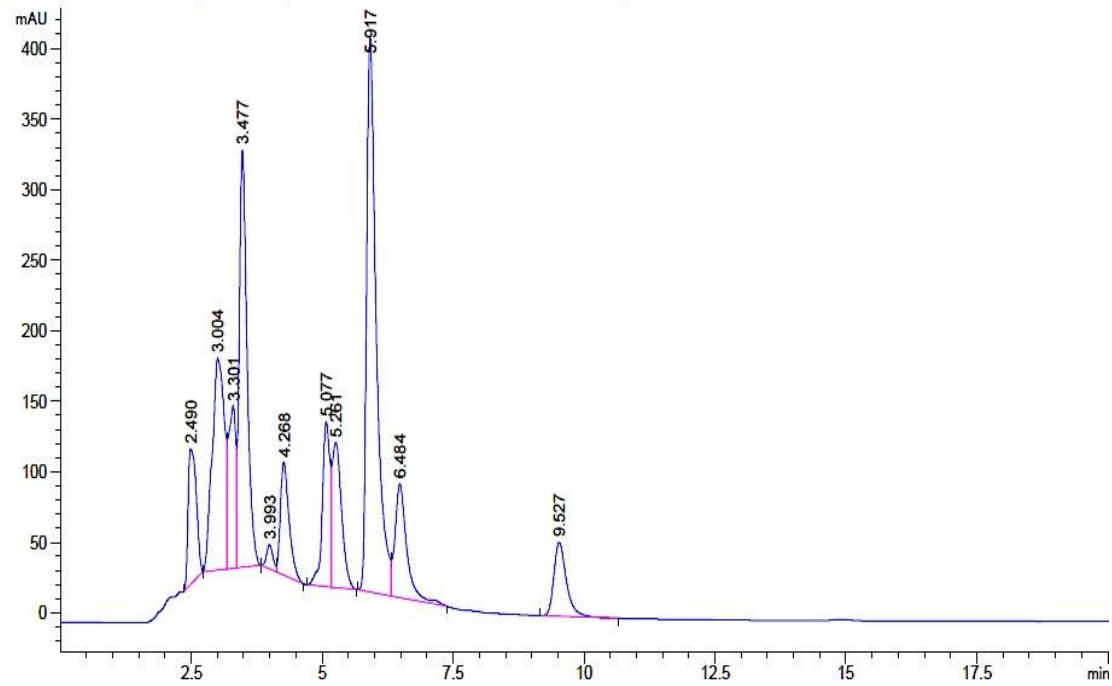


Figure 5.28: Fractions obtained by injecting 250 µl M1 F1 on to a semi-Preparative ACE Excel 5 C-18 amide column, flow rate 5 ml/min, the solvent systems was isocratic mobile phase water: methanol: acetonitrile with a ratio of 20:40:40 (v/v)

M1 F2

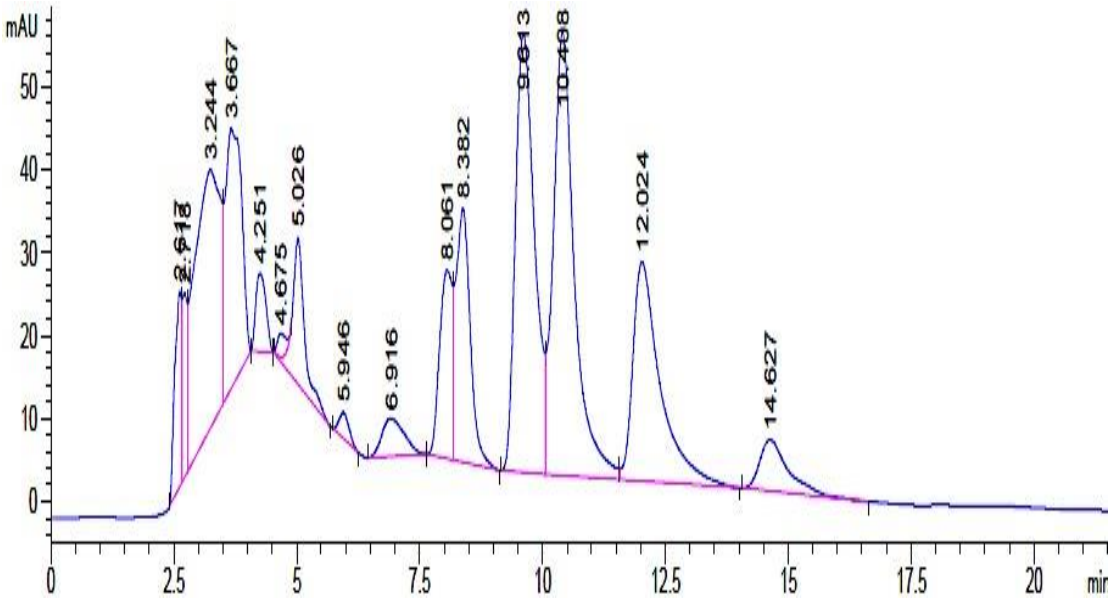


Figure 5.29: Fractions obtained by injecting 250 µl M1 F2 on to semi-Preparative ACE Excel 5 C-18 amide column, flow rate 5 ml/min, the solvent systems was isocratic mobile phase water: methanol 30:70 (v/v).

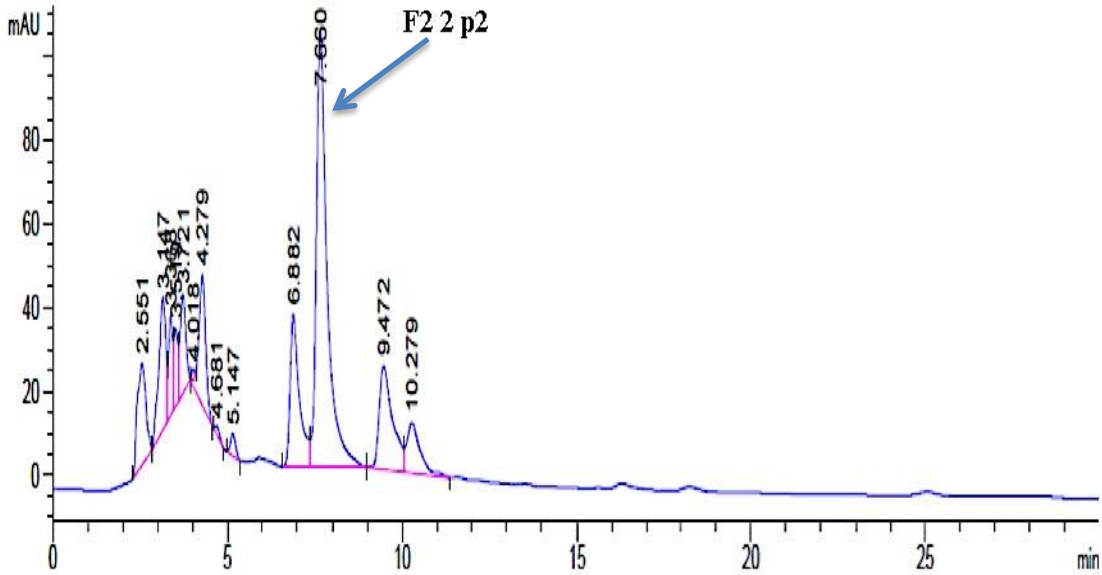


Figure 5.30: Fractions obtained by injecting 250 µl M1 F2 on to semi-Preparative ACE Excel 5 C-18 amide column, flow rate 5 ml/min, the solvent systems was isocratic mobile phase water: methanol: acetonitrile with a ratio of 30:35:35 (v/v)

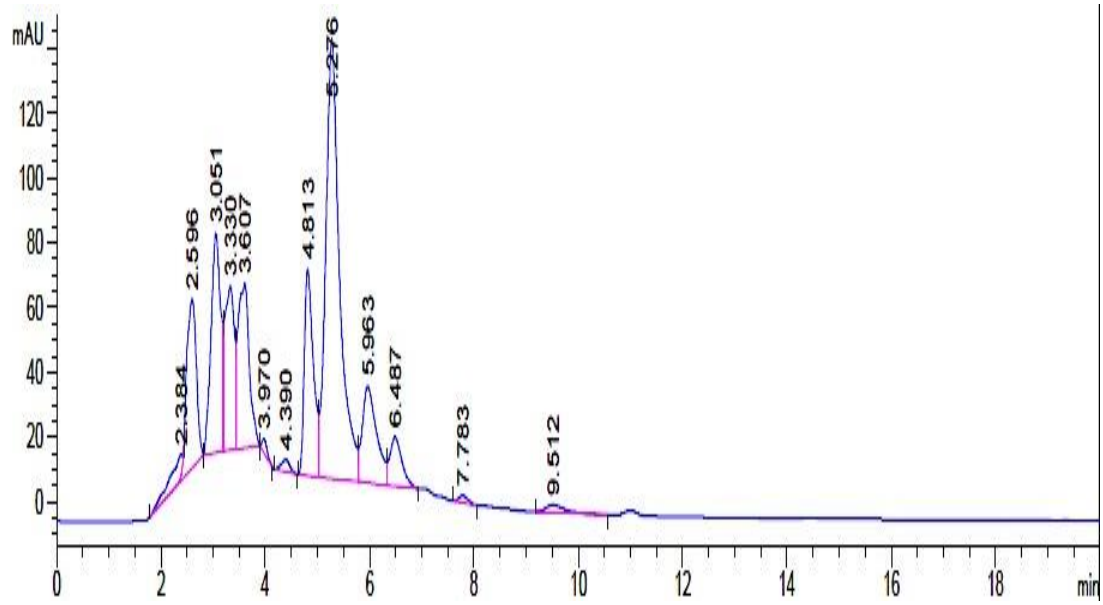


Figure 5.31: Fractions obtained by injecting 250 µl M1 F2 on to semi-Preparative ACE Excel 5 C-18 amide column, flow rate 5 ml/min, the solvent systems was isocratic mobile phase water: methanol: acetonitrile with a ratio of 20:40:40 (v/v)

M1 F3

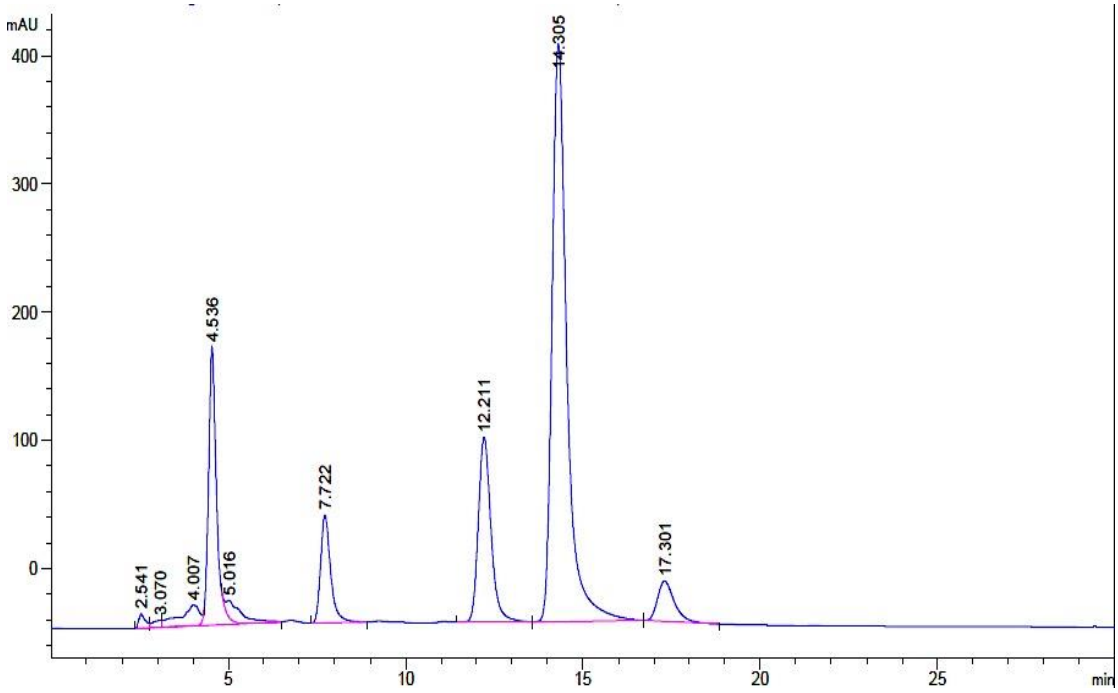


Figure 5.32: Fractions obtained by injecting 250 µl M1 F3 on to semi-Preparative ACE Excel 5 C-18 amide column, flow rate 5 ml/min, the solvent systems was isocratic mobile phase water: methanol 30:70 (v/v).

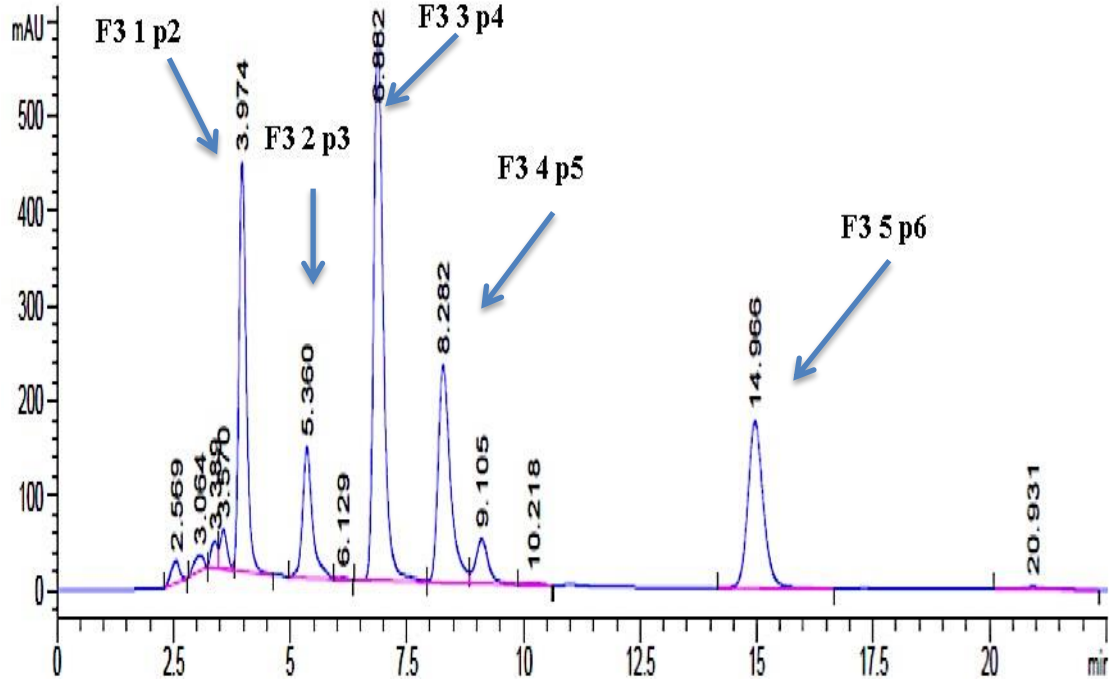


Figure 5.33: Fractions obtained by injecting 250 µl M1 F3 on to semi-Preparative ACE Excel 5 C-18 amide column, flow rate 5 ml/min, the solvent systems was isocratic mobile phase water: methanol: acetonitrile with a ratio of 30:35:35 (v/v)

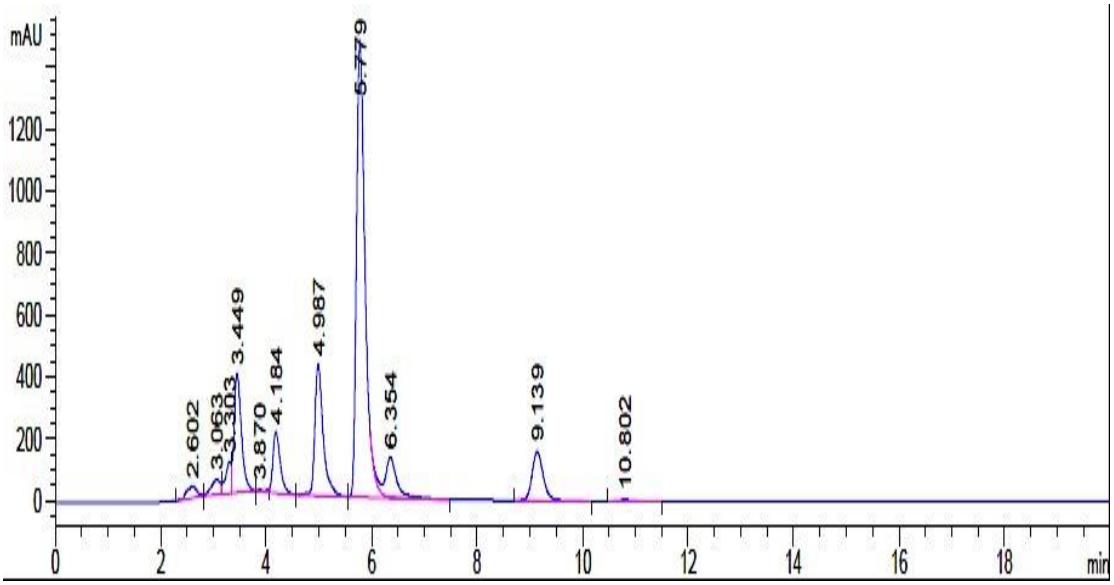


Figure 5.34: Fractions obtained by injecting 250 µl M1 F3 on to semi-Preparative ACE Excel 5 C-18 amide column, flow rate 5 ml/min, the solvent systems was isocratic mobile phase water: methanol: acetonitrile with a ratio of 20:40:40 (v/v)

Each peak with antibacterial activity was collected separately and fractions from different runs were pooled.

M1 F1 (F1 1 p3, F1 2 p4, F1 3 p5 and F1 4 p6)

M1 F2 (F2 2 p2)

M1 F3 (F31 p2, F32 p3, F3 3 p4, F3 4 p5 and F3 5 p6)

The solvent was removed by rotary evaporation and the yellowish brown residue eluted in methanol-d4, each peak was analysed by NMR and the proton spectrum showed that more than one compound was present in M1 F1 (F1 1 p2, F1 2 p3, F1 3 p5 and F1 4 p6), M1 F2 (F2 2 p2) and M1 F3 (F3 1 p2, F3 2 p3 and F3 4 p5), but M1 F3 (F3 3 p4, F3 4 p5 and F3 5 p6) did not need further purification.

To purify the fractions that showed more than one compound on proton spectrum (see Figure 5.73 and Appendix 3.56, 3.65, 3.74, 3.82, 3.88 and 3.96) different solvent system used, solvent system was an isocratic elution using a mobile phase of water: methanol 30:70 (v/v; Figures 5.35-5.41). All the peaks of each fraction were collected separately and assayed for antimicrobial activity. The results showed that there was one peak with antibacterial activity from each fraction, the retention time of the peaks that showed antibacterial activity are summarised in Table 5.6.

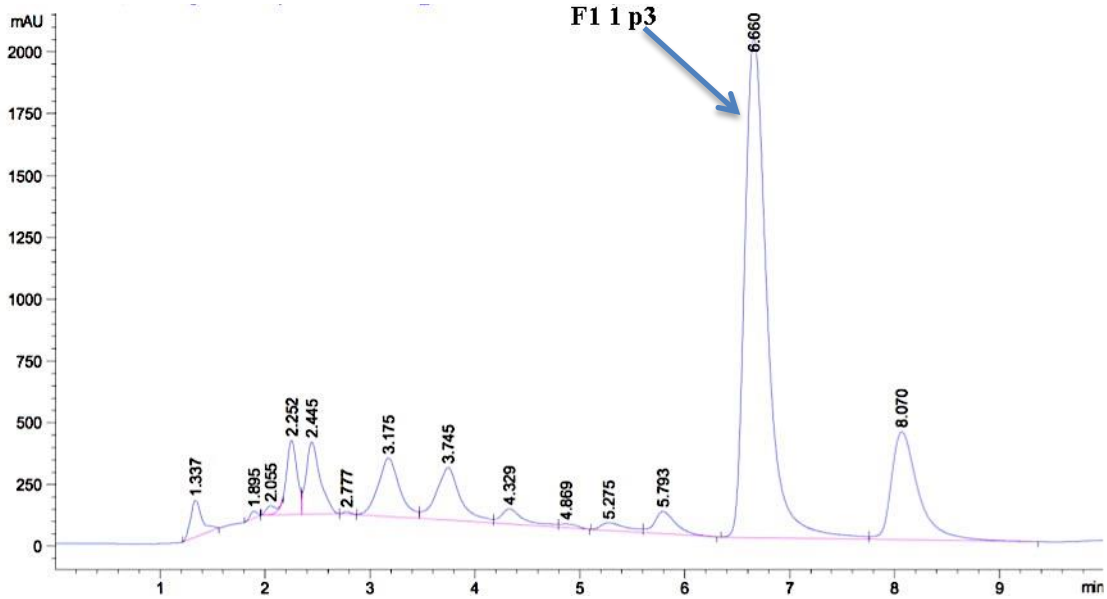


Figure 5.35: Fractions obtained by injecting 250 µl F1 1 p3 on to semi-Preparative ACE Excel 5 C-18 amide column, flow rate 5ml/min, the solvent systems was isocratic mobile phase water: methanol 30:70 (v/v).

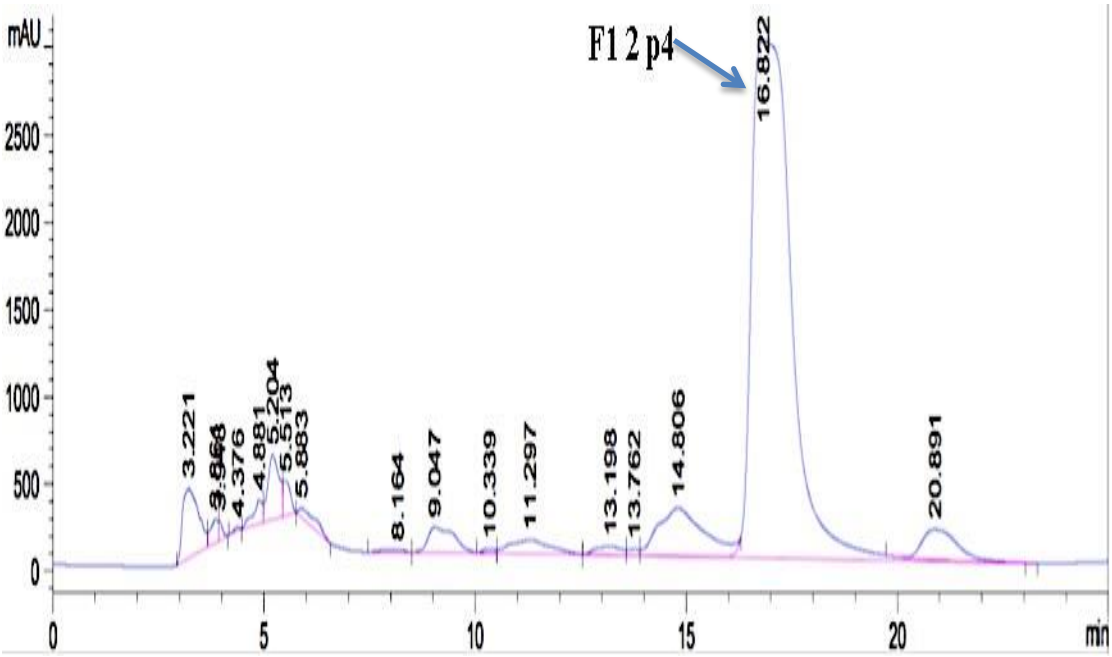


Figure 5.36: Fractions obtained by injecting 250 µl F1 2 p4 on to semi-Preparative ACE Excel 5 C-18 amide column, flow rate 5ml/min, the solvent systems was isocratic mobile phase water: methanol 30:70 (v/v).

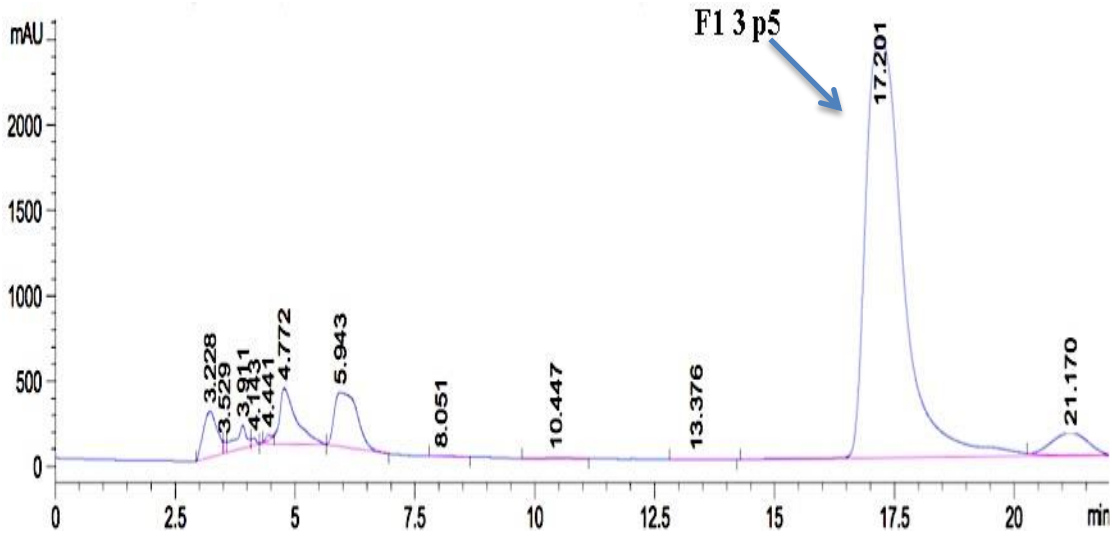


Figure 5.37: Fractions obtained by injecting 250 µl F1 3 p5 on to semi-Preparative ACE Excel 5 C-18 amide column, flow rate 5ml/min, the solvent systems was isocratic mobile phase water: methanol 30:70 (v/v).

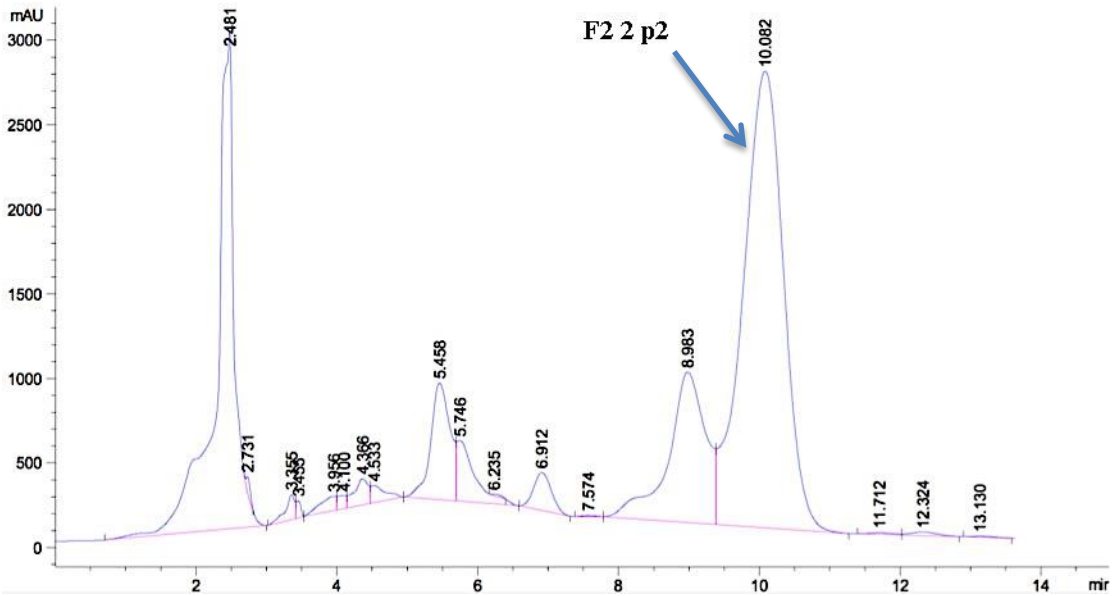


Figure 5.38: Fractions obtained by injecting 250 µl F2 2 p2 on to semi-Preparative ACE Excel 5 C-18 amide column, flow rate 5ml/min, the solvent systems was isocratic mobile phase water: methanol 30:70 (v/v).

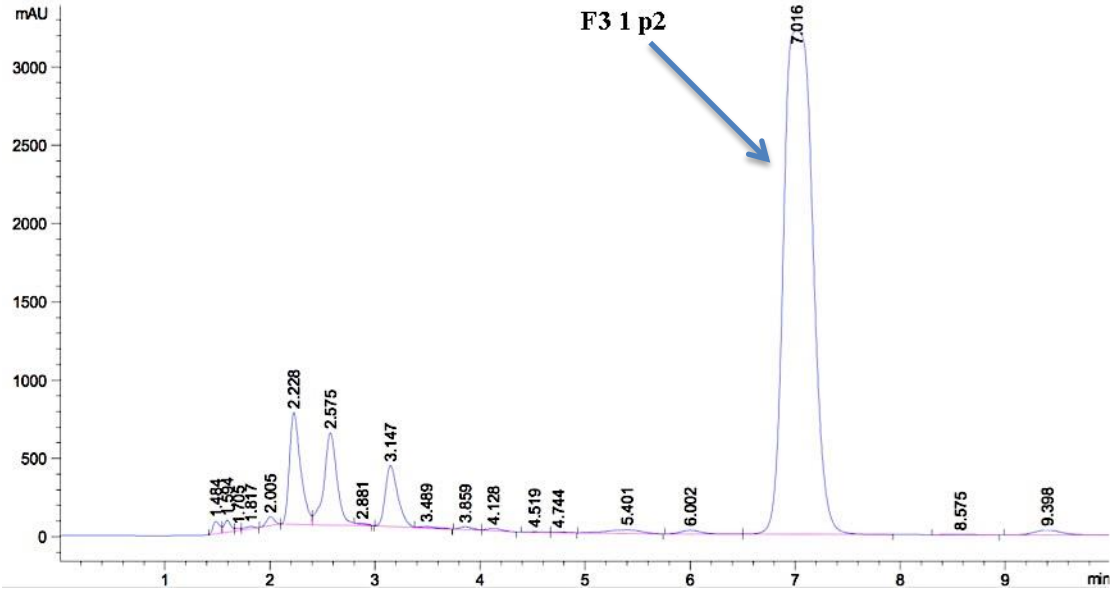


Figure 5.39: Fractions obtained by injecting 250 µl F3 1 p2 on to semi-Preparative ACE Excel 5 C-18 amide column, flow rate 5ml/min, the solvent systems was isocratic mobile phase water: methanol 30:70 (v/v).

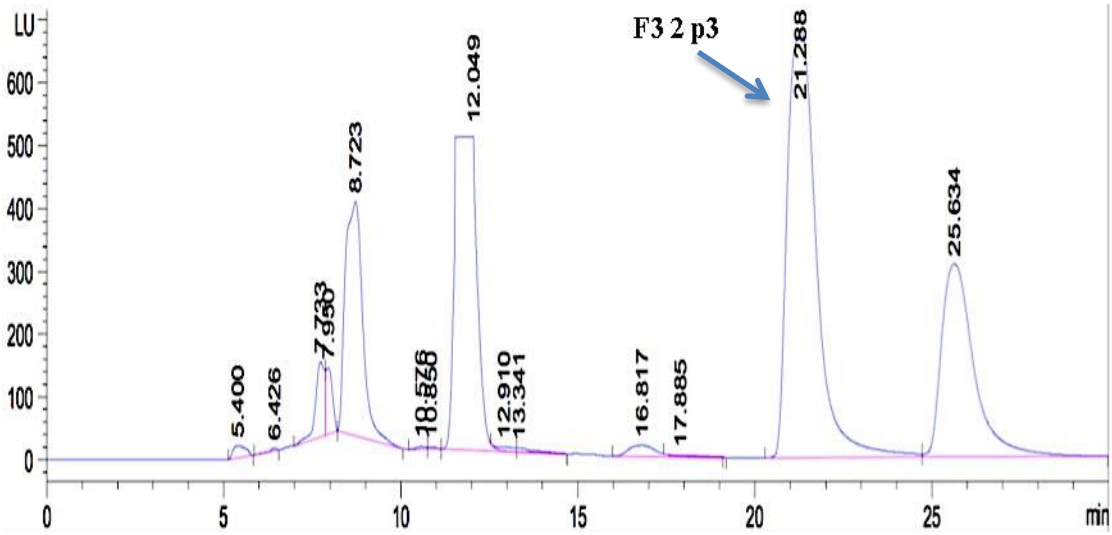


Figure 5.40: Fractions obtained by injecting 250 µl F3 2 p3 on to semi-Preparative ACE Excel 5 C-18 amide column, flow rate 5ml/min, the solvent systems was isocratic mobile phase water: methanol 30:70 (v/v).

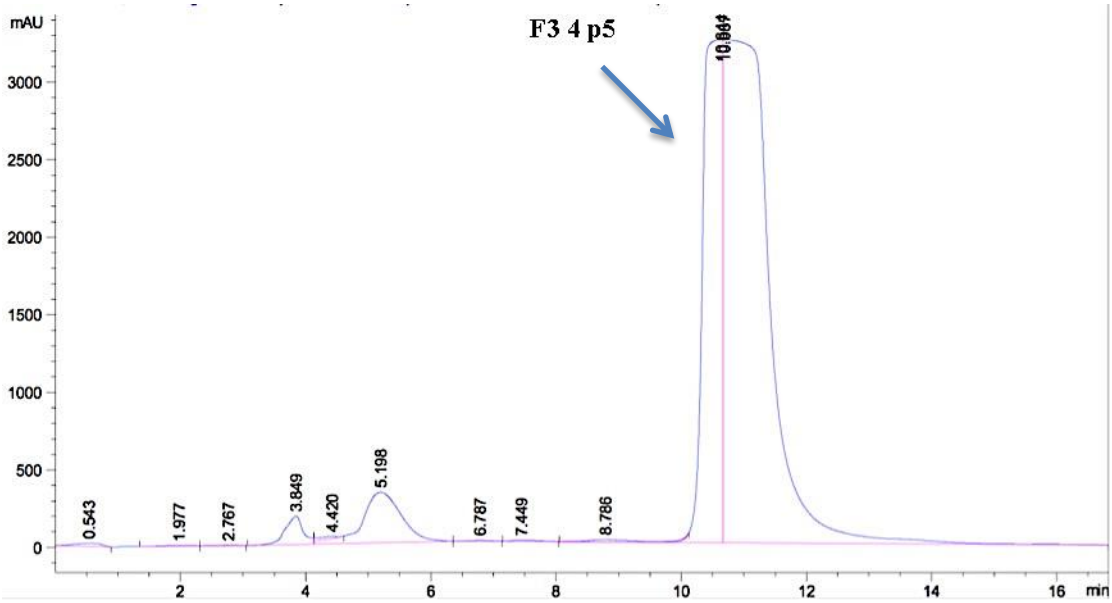


Figure 5.41: Fractions obtained by injecting 250 µl F3 4 p5 on to semi-Preparative ACE Excel 5 C-18 amide column, flow rate 5ml/min, the solvent systems was isocratic mobile phase water: methanol 30:70 (v/v).

Table 5.6: Summary of retention time for the peaks with antibacterial activity from the fractions from M1 11 extracts that showed more than one compound on proton spectrum

Peak	F1 1 p3	F1 2 p4	F1 3 p5	F2 2 p2	F3 1 p2	F3 2 p3	F3 4 p5
Retention time	6.660 min	16.822 min	17.201 min	10.082 min	7.016 min	21.288 min	10.684 min

The HPLC was repeated for all the extracts and the active fractions pooled, concentrated by rotary evaporation and weighed The following amounts were obtained: M1 F1 (F1 1 p3 0.9 mg, F1 2 p4 1.8 mg, F1 3 p5 1.5 mg and F1 4 p6 3 mg), M1 F2 (F2 2 p2 0.8 mg) and M1 F3 (F3 1 p2 0.9 mg, F3 2 p3 0.9 mg, F3 3 p4 3 mg, F3 4 p5 8 mg and F3 5 p6 4 mg). The purification scheme is summarized in Table 5.7. Further analysis of the fractions including NMR, IR and accurate MS was done to obtain the chemical elements of the compound and the possible structure of the antibiotics.

Table 5.7: Summary of purification of the antibiotics for M1 11 extraction by HPLC (each step of the purification all the fractions collected separately and tested for antimicrobial activity)

Crude extract	First step of the purification, the solvent systems was water: acetonitrile 33:67 (v/v)	Second step of the purification, the solvent systems water: methanol: acetonitrile 30:35:35 (v/v)	Third step of the purification, the solvent systems was water: methanol 30:70 (v/v)
Injecting M1 11 crude extract on to reverse phase HPLC	F1 at RT 8.547, not pure fraction	F1 1 p3 at RT 4.162 min, Semi-pure fraction	F1 1 p3 (0.9 mg) at RT 6.660 min Pure fraction
		F1 2 p4 at RT 7.570 min, Semi-pure fraction	F1 2 p4 (1.8 mg) at RT 16.822 min Pure fraction
		F1 3 p5 at RT 9.246 min, Semi-pure fraction	F1 3 p5 (1.5 mg) at RT 17.201 min Pure fraction
		F1 4 p6 (3 mg) at RT 17.529 min, did not need further purification	
	F2 at RT 16.688, not pure fraction	F2 p2 at RT 7.660 min, semi-pure fraction	F2 p2 (0.8 mg) at RT 10.082 min Pure fraction
	F3 at RT 26.271, not pure fraction	F3 1 p2 at RT 3.974 min, semi-pure fraction	F3 1 p2 (0.9 mg) at RT 7.016 min Pure fraction
		F3 2 p3 at RT 5.360 min, semi-pure fraction	F3 2 p3 (0.9 mg) at RT 21.288 min
		F3 3 p4 (3 mg) at RT 6.882 min, did not need further purification	
		F3 4 p5 at RT 8.282 min, semi-pure fraction	F3 4 p5 (8 mg) at RT 10.684 min Pure fraction
		F3 5 p6 (4 mg) at RT 14.966 min, did not need further purification	

5.2.2 Identification of Chemical Structures

5.2.2.1 Es1 Fractions

NMR: Spectroscopy and Interpretation

All NMR measurements were performed on a Bruker Ultra-shield Avance AMX 400 MHz spectrometer as described in section 2.17.4

Es1 Fraction 6

The NMR results of Es1 Fraction 6 are shown in Figures 5.43-5.48 Appendix 3.1 to 3.13. ^{1}H NMR showed peaks at 7.75ppm, 7.05ppm and 6.90ppm which are characteristic of protons found in aromatic rings (Figure 5.43), the 2D g-COSY showed that the signals at 7.75ppm and 6.90ppm were adjacent to each other on the ring system, and is consistent with the peak splitting seen as a doublet (number of peaks = n+1 where n is the number of protons on the adjacent carbon; Figure 5.44). The signal at 7.05ppm was a singlet so has no adjacent carbons with protons and was not correlated with other protons on the COSY. The alcohol groups are not present due to deuterium exchange of the hydroxyl group. The signal at 3.4ppm shows hydrogen deuterium oxide (DOH) produced by the deuterium exchange and the signal at 2.50ppm was from the solvent (Figure 5.43). The ^{13}C NMR had seven signals, the signal at 172.6ppm corresponding to a carboxyl group and that at 159.6ppm with an enol group. The five remaining signals 131.8ppm, 127.5ppm, 122.8ppm, 114.6ppm, 115.9ppm all fall into the aromatic region and, due to the low number of signals present, indicated that the molecule had some symmetry involved as the signals would overlap and be seen as a single signal, or due to the low concentration of the molecule some peaks may not be present (Figure 5.47). The JMOD ^{13}C NMR showed that the signals at 172.6ppm, 159.6ppm, 122.8ppm and 114.6ppm were ether non protonated carbons or di-protonated carbons, as the splitting from the 1H NMR indicates the liklihood of these being di-protonated is minimal (Figure 5.48).

The 2D g-HMQC and 2D g-HMBC NMR showed that the linked signals at 7.75ppm and 6.90ppm on the ^{1}H NMR were associated with the signals 131.8ppm and 115.9ppm respectively (Figures 5.45 and 5.46). The difference in the ^{1}H NMR ppm is due to the deshielding effect of a hydroxyl group in addition to the anisotropy effect of the aromatic ring which indicated that the 7.75 ppm proton was next to a carbon

with a hydroxyl group attached. This indicated an aromatic ring which has two adjacent protons with no protons on the next carbons along in the ring. The ^{1}H NNR signal at 7.05ppm was correlated with the ^{13}C NMR signal at 127.5ppm which is indicative of a aromatic carbon with a hydroxyl group attached to the ^{1}H NMR 7.05pmm.

The FTIR spectrum is shown in Figure 5.49 and showed transmission bands associated with alcohol groups at 3300 cm^{-1}, aromatic groups at 2452 cm^{-1}, carbonyl group at 1602 cm^{-1} and enol / ether group at 1510 cm^{-1}. These bands correlate with the structure suggested by the NMR data (Figures 5.43-5.48).

Using this information gained from the FTIR and NMR (Table 5.8) it was possible to build a picture of the molecule. This gave a molecule with the chemical formula $C_{15}H_{10}O_6$ which has a mass of 286.05 da. The mass spectrum of the compound showed a peak at 287.08 which corresponds to the $[M+H]^-$ ion which helps to confirm the structure proposed (3, 4', 7, 8 tetrahydroxyflavone; Figure 5.45).

Table 5.8: Summarised of NMR and FTIR data.

Atom Number	1H NMR	13C NMR	FTIR
1	-	-	1510
2	-	122.8	1510
3	-	159.67	1510
4	-	172.63	1602
5	-	127.53	2452
6	7.05	114.6	2452
7	-	127.53	3300
8	-	127.53	3300
9	7.05	114.6	2452
10	-	127.53	2452
1*	-	122.8	2452
2*	6.9	115.9	2452
3*	7.75	131.8	2452
4*	-	122.8	3300
5*	7.75	131.8	2452
6*	6.9	115.9	2452

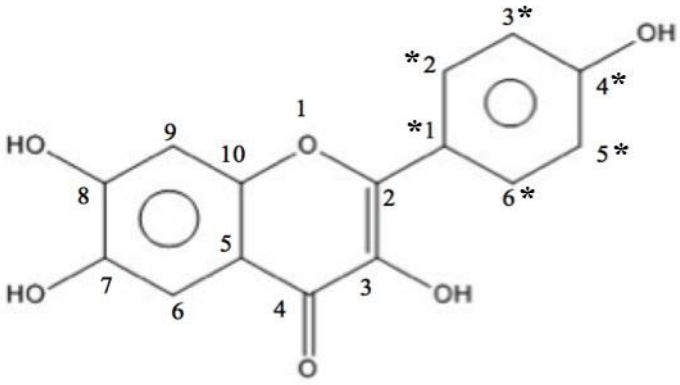


Figure 5.42: The chemical structure of compound Es1 F6 build by NMR, FTIR and MS ($C_{15}H_{10}O_6$)

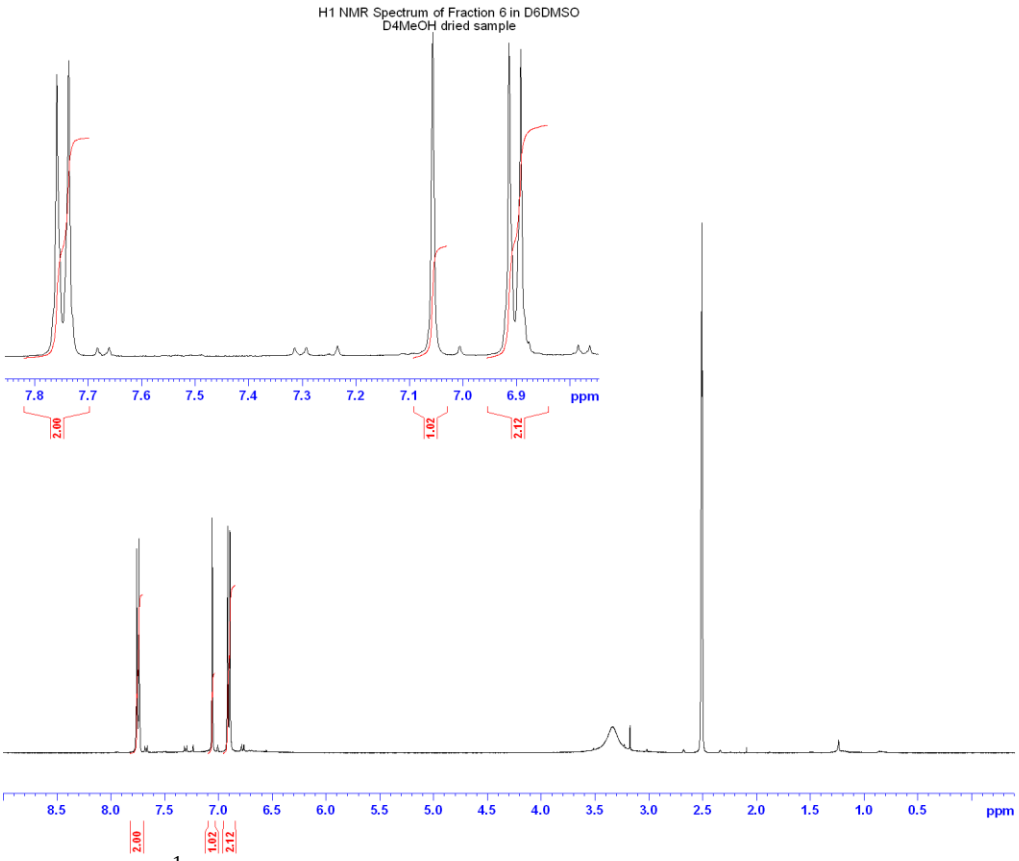


Figure 5.43: ^{1}H NMR Spectrum of ES1 F6 in D6DMSO+TMS

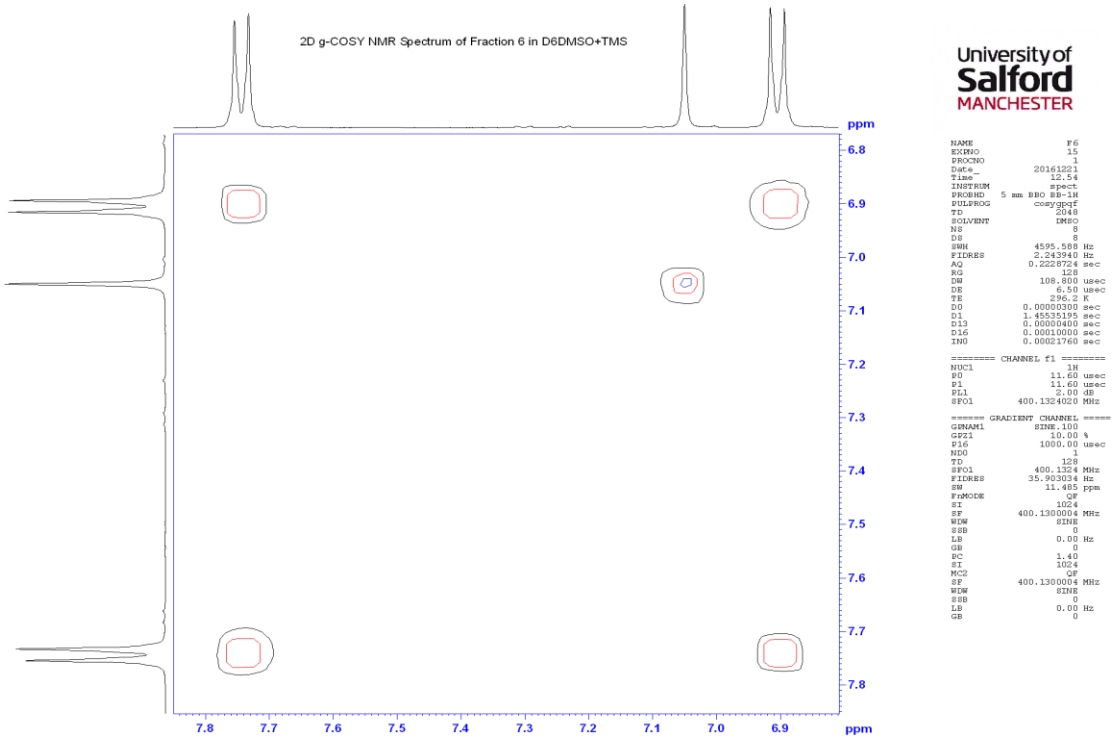


Figure 5.44: 2D g-COSY NMR Spectrum of Es1 F6 (expansion) in D6DMSO+TMS

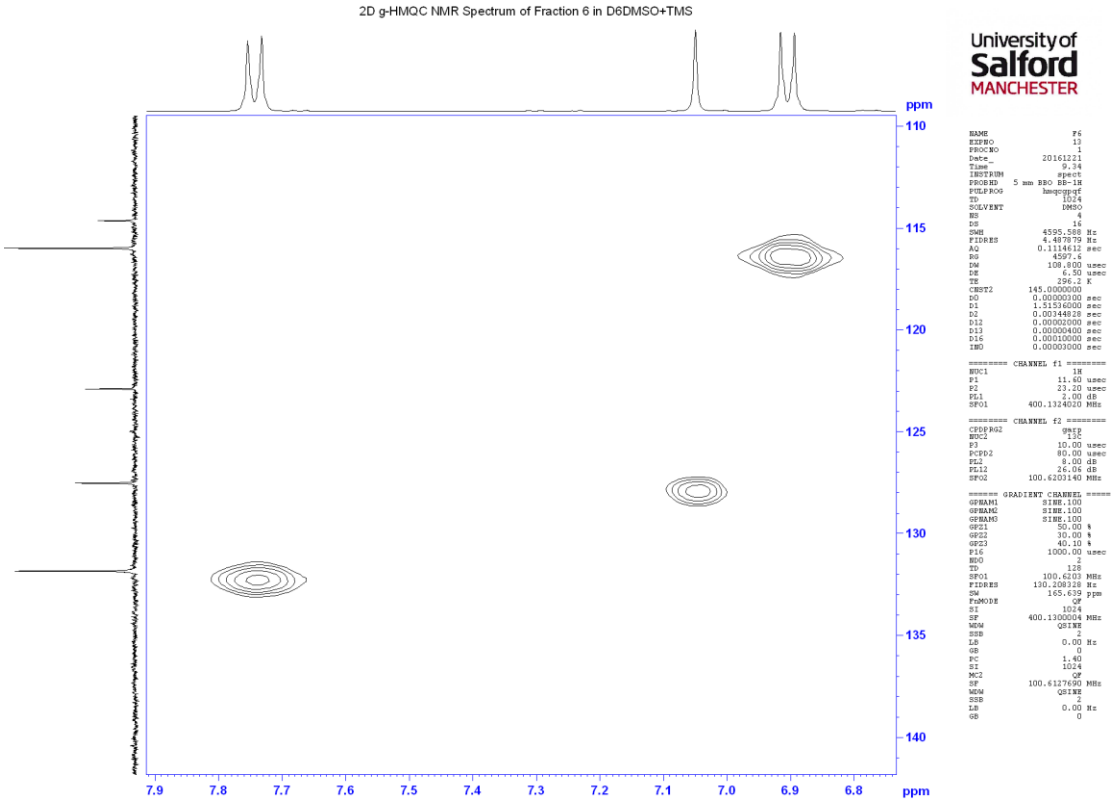


Figure 5.45: 2D g-HMQC NMR Spectrum of Es1 F6 (expansion) in D6DMSO+TMS

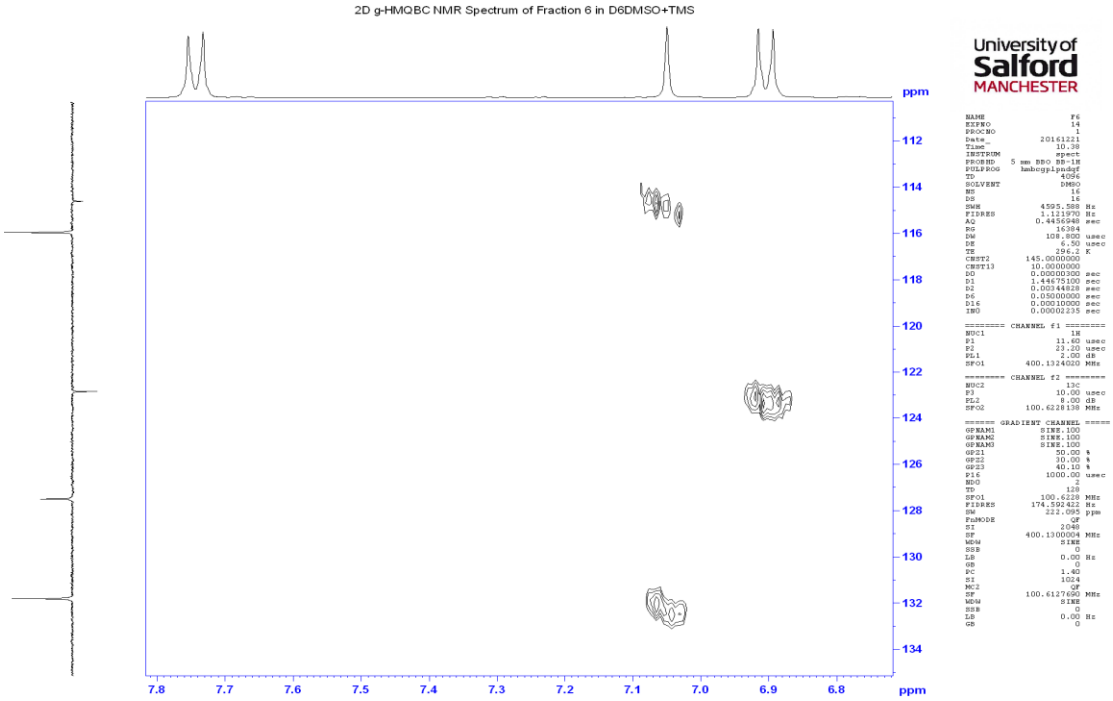


Figure 5.46: 2D g-HMBC NMR Spectrum of Es1 F6 (expansion) in D6DMSO+TMS

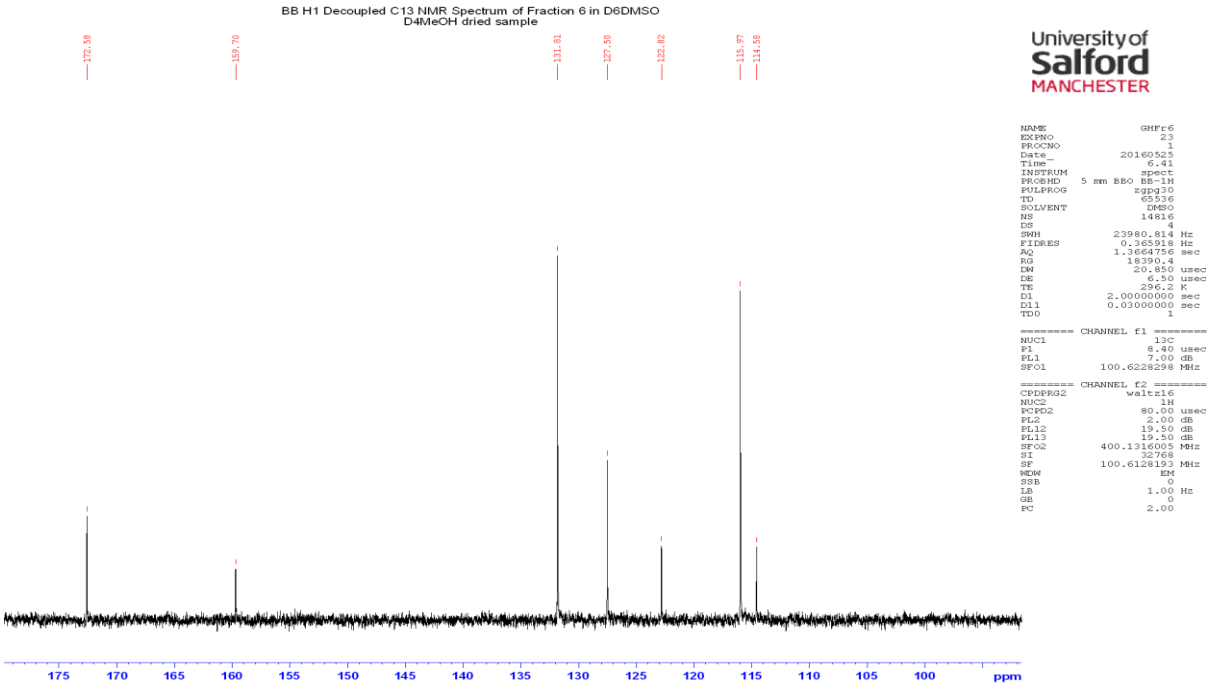


Figure 5.47: BB H1 Decoupled C13 NMR Spectrum of ES1 F6 (expansion) in D6DMSO

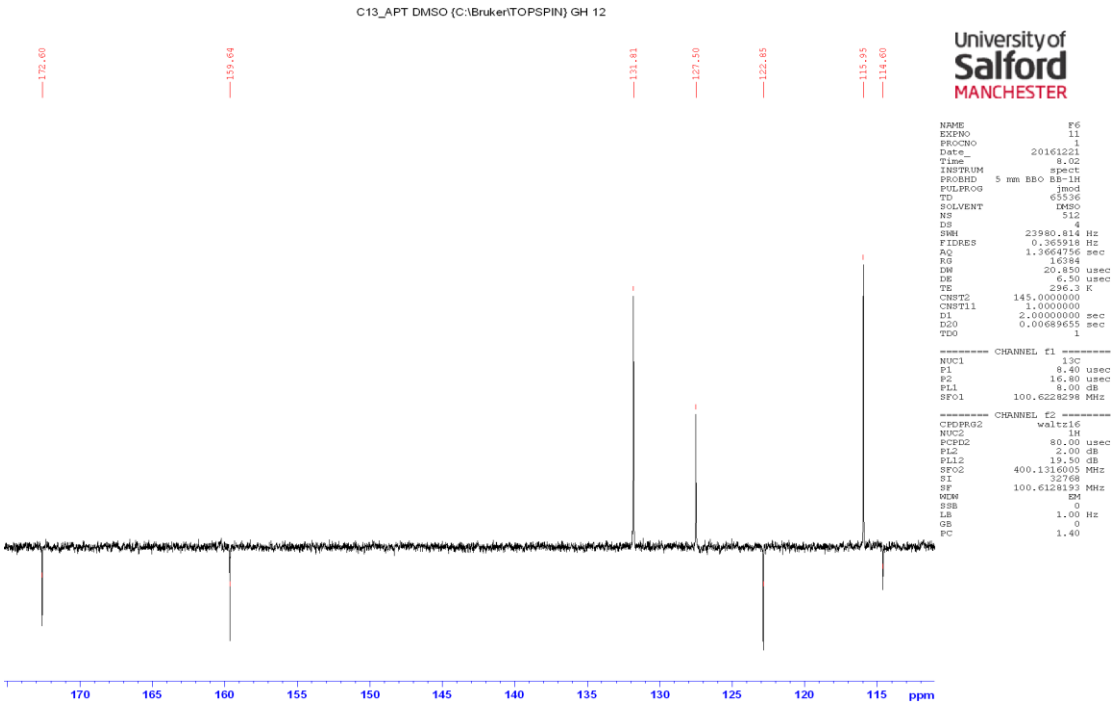


Figure 5.48: JMOD C13 Spectrum of Es1 F6 (expansion) in D6DMSO+TMS

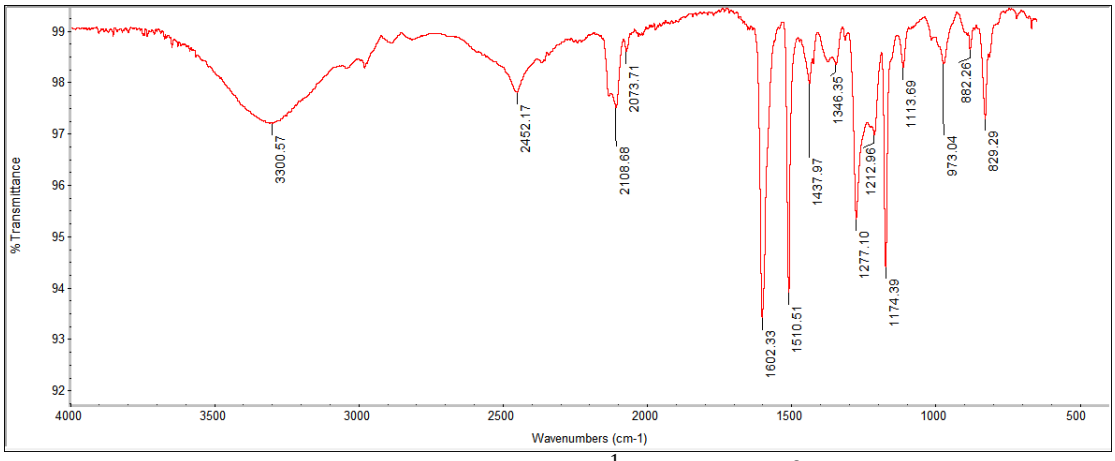


Figure 5.49: The FTIR spectrum (ATR) 4 cm $^{-1}$ (16 Scans) of F6

Mass Spectroscopy (MS) of the compound Es1 F6.

Samples of Es1 F6 were sent to the Cambridge University Chemical Laboratory for accurate mass analysis and the results are shown in Figure 5.50 and Appendix 3.14 to 3.16 (similar MS results were obtained from LC MS at University of Salford University Appendix 3.17). The results gave a MZ of 286. The results are summarised below. All the data supports the structure shown in Figure 5.51 (3, 4′, 6, 7-tetrahydroxyflavone.

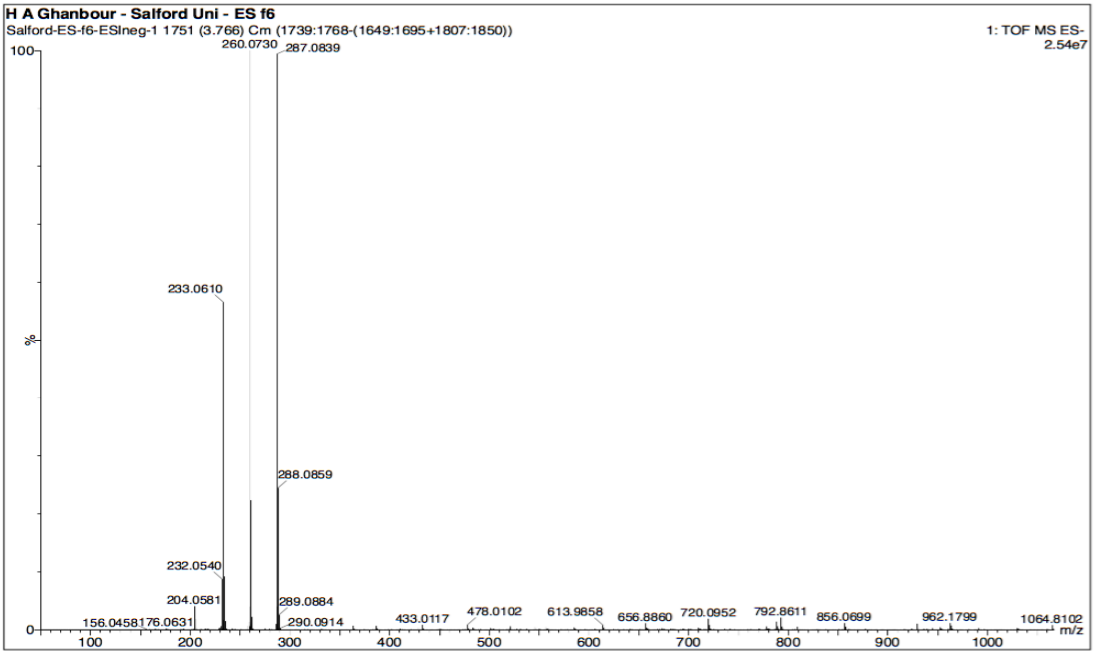


Figure 5.50: The negative ion spectrum from LC MS-TOF of Es1 F6

Summary of spectral data of compound Es1 F6

^{1}H-NMR (400 MHz, CD_3OD): δ H 7.75 doublet of doublet (d of d), 7.06 singlet (s) and 6.9 doublet of doublet (d of d)

^{13}C NMR (400 MHz, CD3OD): δ ^{13}C 172.58, 159.70, 131.81, 127.50, 122.82, 115.97 and 114.58

FTIR spectra (ATR) 4 cm $^{-1}$ (16 Scans): 3300 broad (br), 2452 small broad (sbr), 2108 medium sharp (ms), 1602 sharp (s), 1510 sharp (s), 1436 small medium sharp (sms), 1346 small broad (sbr), 1277 sharp (s), 1174 sharp (s), 1113 small sharp (ss), 973 small broad (sbr), 882 small sharp (ss) and 829 small sharp (ss)

MS: MZ=287 **(**$M+H)^-$

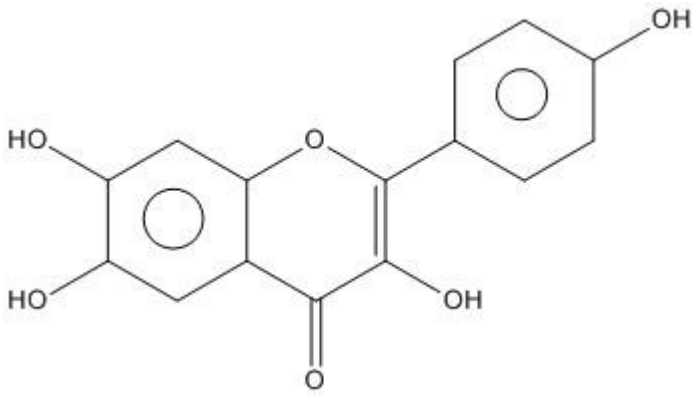


Figure 5.51: The confirmed chemical structure of compound Es1 F6 ($C_{15}H_{10}O_6$, MW MZ=287 (M+H)-).

Es1 Fraction 3/1

The NMR results of Es1 Fraction 3/1 are shown in Figures 5.52 and 5.53, Appendix 3.18. The ^{1}H NMR spectrum showed a strong signal in the aromatic region (between 6.8 to 8 ppm; Figure 5.52). 2D g- COSY spectra showed two different aromatic rings not next to each other (Figure 5.53). The NMR spectrum showed peak at 6.9ppm (2H), 7.75ppm (2H) and 6.9ppm (1H), 7.15ppm (1H), 7.4 ppm (1H) showing presence of two benzene rings, 1,4-disubstituted and 1,2,4-trisubstituted respectively (Figure 5.52). This was confirmed by the 2D COSY spectrum. The ^{1}H NMR and 2D g-Cosy spectra did not show protons on the alcohols due to exchange with deuterium (Figures 5.52 to 5.53). The FTIR spectra showed alcohol group (3397 cm $^{-1}$), aromatic group (2255 cm $^{-1}$), carbonyl group (1659 cm $^{-1}$) and enol groups (1437 cm $^{-1}$; Figure 5.54). The range of MS showed too many signal on positive and negative mode due the compound not being stable at the temperature used (Appendix 3.19 to 3.21) and the MS of the compound showed a peak at 319.07 which corresponds to the [M+H]$^{-}$ ion in negative mode (Figure 5.55).

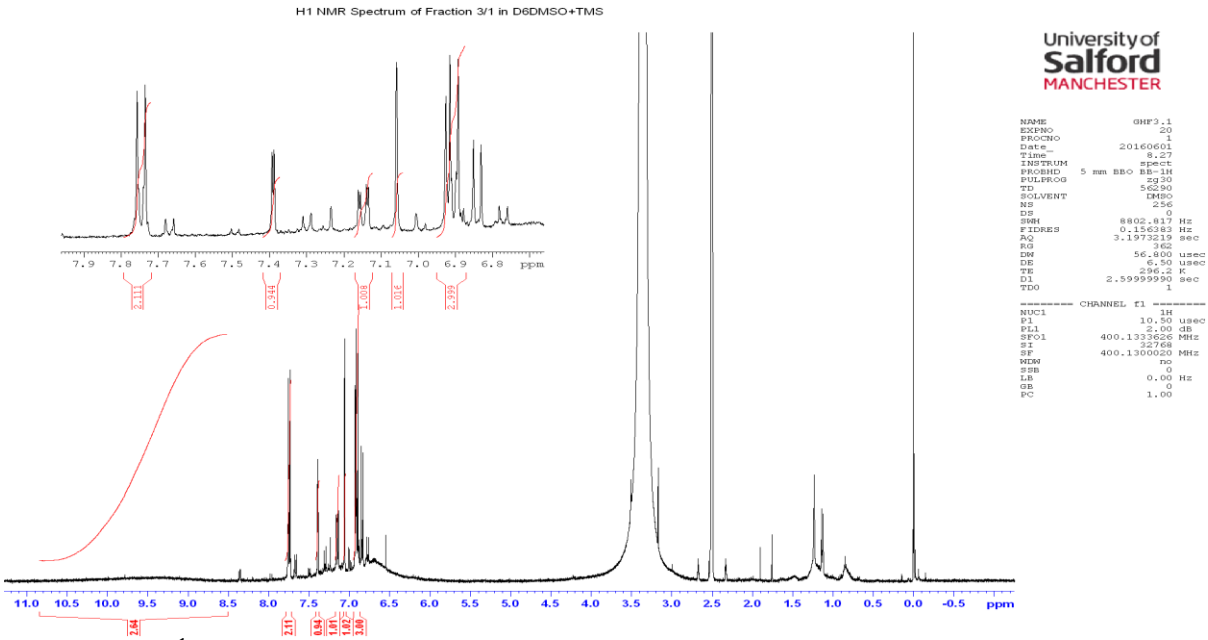


Figure 5.52: ^{1}H NMR Spectrum of Es1 F3/1 in D_6DMSO+TMS

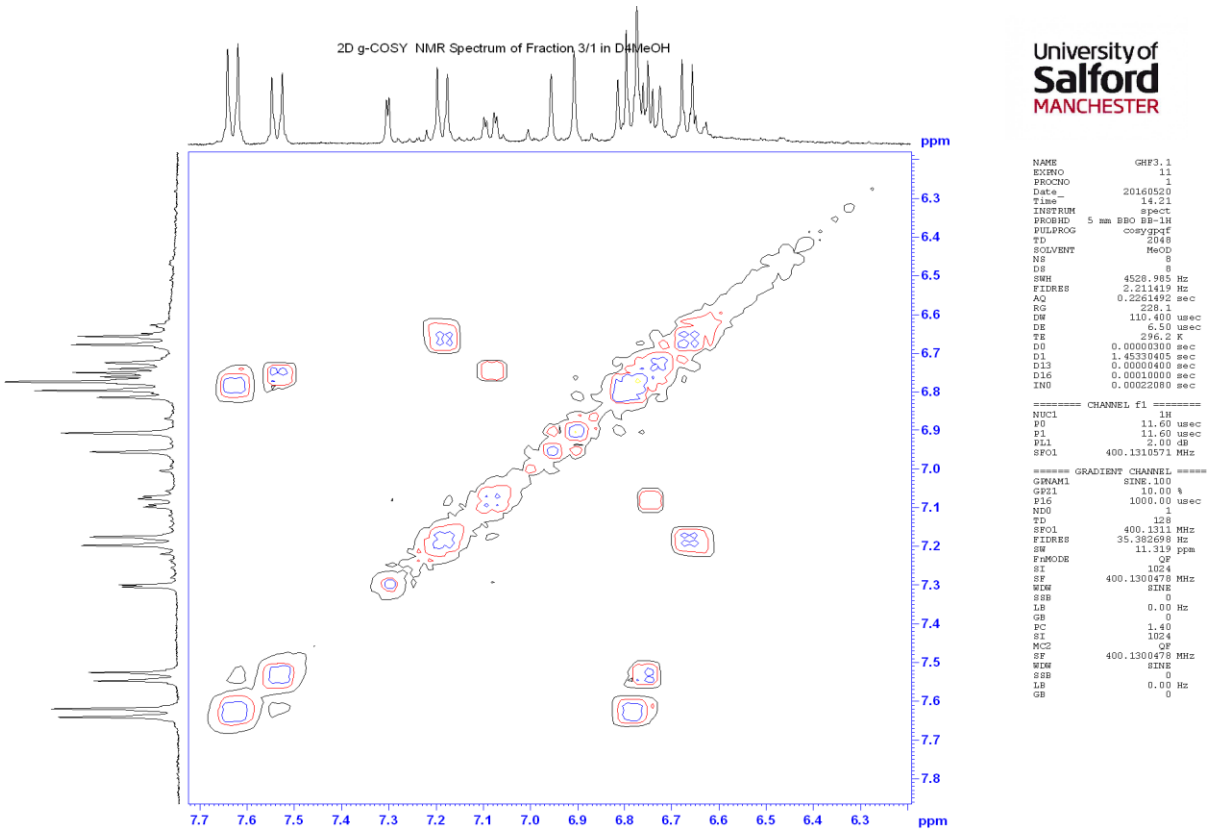


Figure 5.53: 2D g-COSY NMR Spectrum of Es1 F3/1 (expansion) in CD_3OD

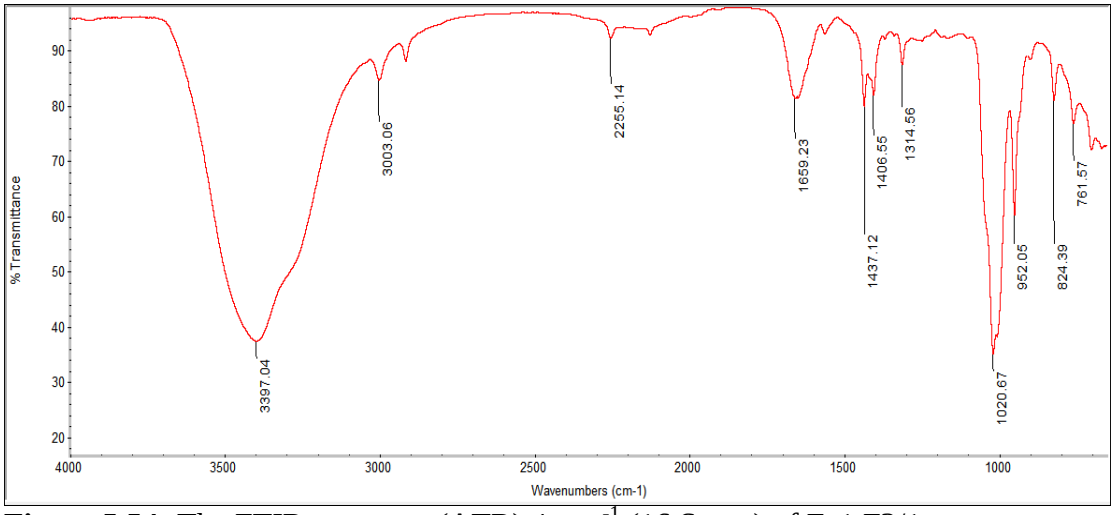


Figure 5.54: The FTIR spectrum (ATR) 4 cm^{-1} (16 Scans) of Es1 F3/1

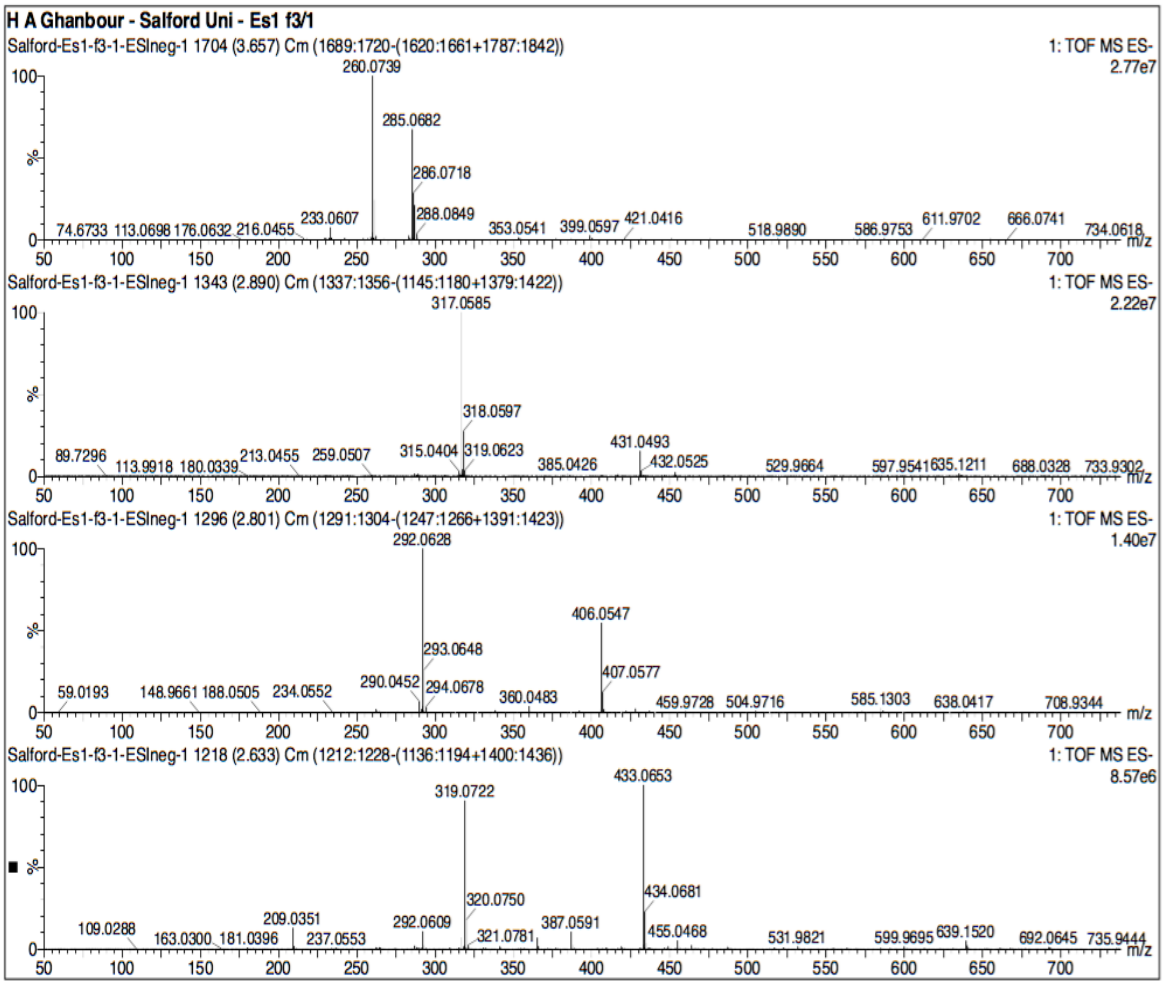


Figure 5.55: The negative ion spectrum from LC MS-TOF of Es1 F3/1

Summary of spectral data of compound Es1 F3/1

^{1} H-NMR (400 MHz, CD_3OD): δ H 7.75(d of d), 7.39(d), 7.15(d of d), 7.06(s), 6.92(s), 6.91(d of d) and 6.84(d)

^{13}C NMR (400 MHz, CD_3OD):

Due limited amount of material ^{13}C NMR was not be obtained.

FTIR spectra (ATR) 4 cm $^{-1}$ (16 Scans): 3397 (br), 3003 (sms), 2255 (sms), 1659 (s), 1406 (s), 1314 (ss), 1020 (s), 952 (s), 824 (ss) and 761(ss)

MS: M/Z=319 **(**M-H)$^{-}$

The FTIR, NMR and MS data are consistent with the structure shown in Figure 5.56 (2′, 4′, 6′, 3, 6, 8- hexahydroxyflavone).

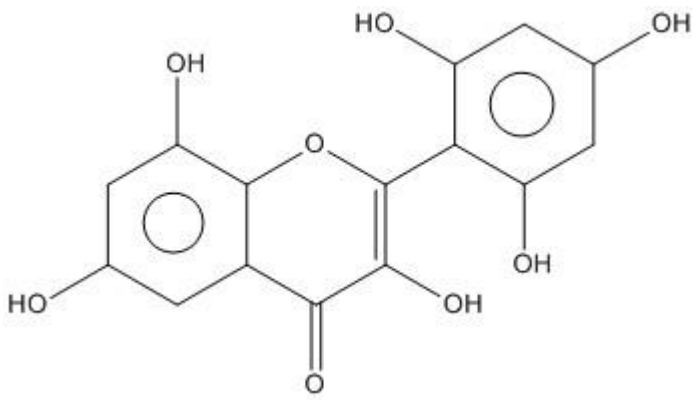


Figure 5.56: The proposed chemical structure of compound Es1 F3/1 ($C_{15}H_{10}O_8$, MW MZ=318 (M-H)-).

Es1 Fraction 3/3

The NMR results of Es1 Fraction 3/3 are shown in Figure 5.57 and 5.58 Appendix 3.22 to 3.24. The ^{1}H NMR Spectrum was very similar to the one obtained from Es1 F6, the ^{1}H NMR spectrum showed two aromatic rings (Figure 5.57). The ^{1}H NMR spectrum and 2D COSY had peaks at 7.63ppm (2H), 6.78pp (2H) and 6.9ppm (1H), indicating presence of possibly two benzene rings, 1,4-disubstituted and pent substituted (or olefinic hydrogen) respectively (Figure 5.57 and 5.58). The 2D g-COSY showed a similar spectrum to fraction Es1 F6 (Figure 5.58). Unfortunately the ^{13} C spectrum was too weak due to lack of material and did not show all signals (Appendix 3.23). The FTIR spectrum showed the same groups as Es1 F6 (Figure 5.59). The range of MS showed too many signal on positive and negative mode (Appendix 3.25 to 3.27) and the MS of the compound showed a peak at 287 which corresponds to the $[M+H]^{-}$ ion in negative mode (Figure 5.60).

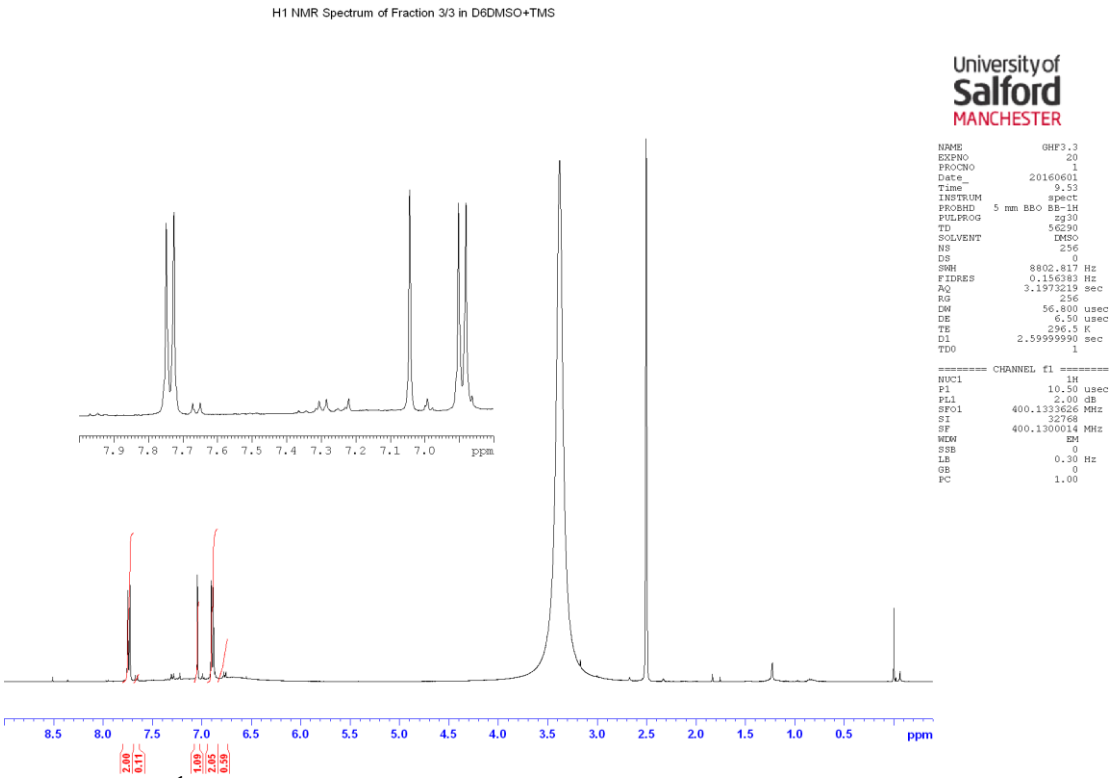


Figure 5.57: ^{1}H NMR Spectrum of Es1 F3/3 in D_6DMSO+TMS

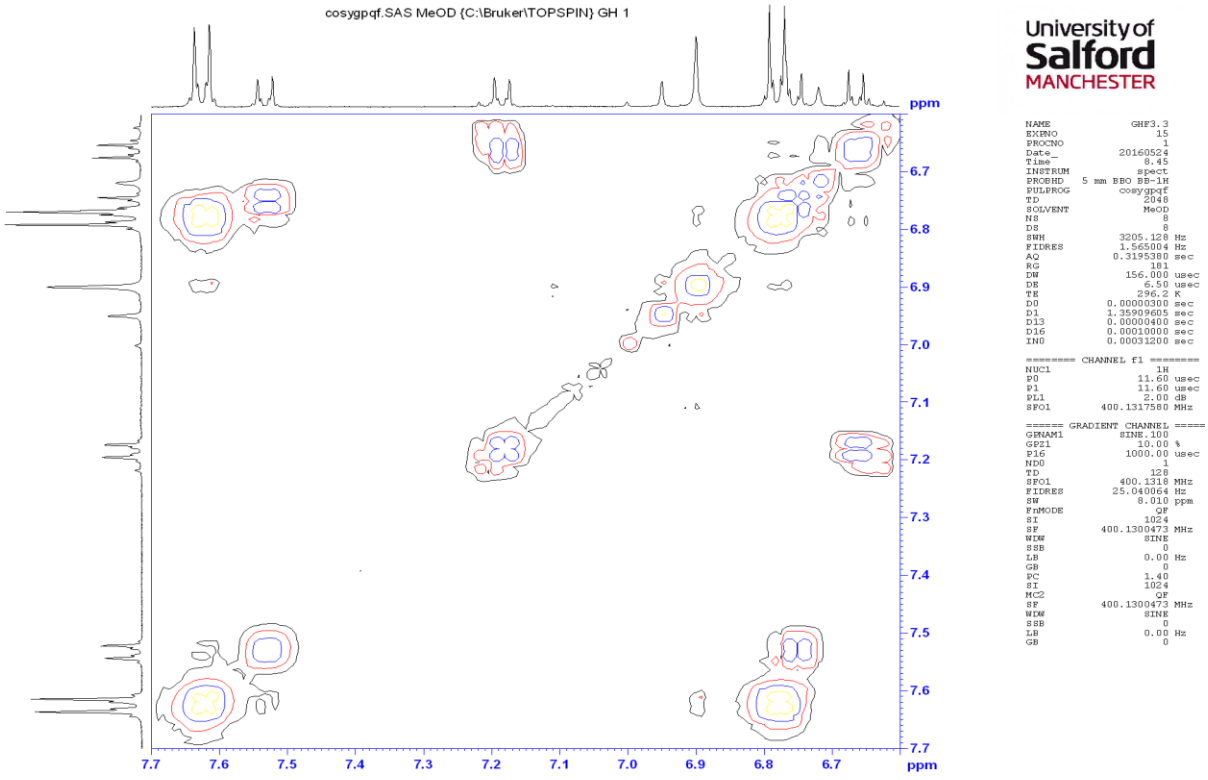


Figure 5.58: 2D g-COSY NMR Spectrum of Es1 F3/3 (expanded) in CD_3OD

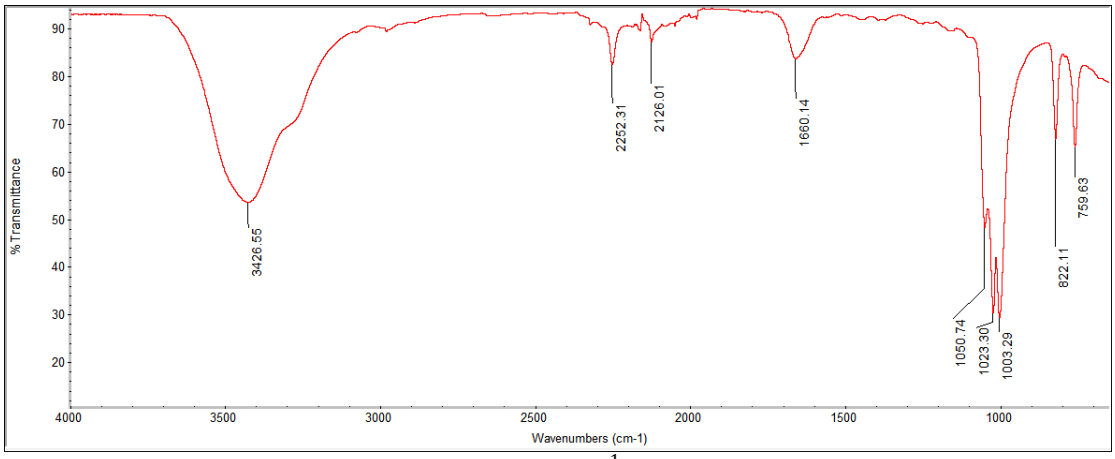


Figure 5.59: The FTIR spectrum (ATR) 4 cm $^{-1}$ (16 Scans) of Es1 F3/3

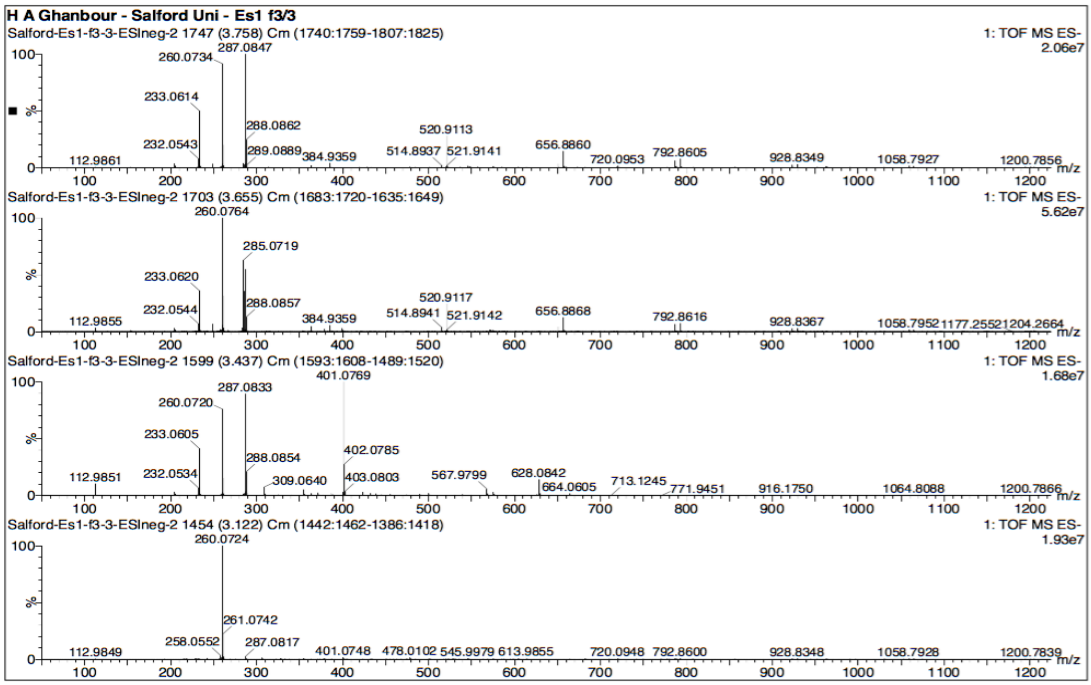


Figure 5.60: The negative ion spectrum from LC MS-TOF of Es1 F3/3

Summary of spectral data of compound Es1 F3/3

^{1}H-NMR (400 MHz, CD_3OD): δ H 7.63(d of d), 6.9(s) and 6.78(d of d)

^{13}C NMR (400 MHz, CD_3OD):

Due limited amount of material ^{13}C NMR was not obtained

FTIR spectra (ATR) 4 cm $^{-1}$ (16 Scans): 3426 (br), 2252 (ss), 2126 (ss), 1660 (sbr), 1050(ss), 1023(s), 1003(s), 822 (ss) and 759 (ss)

MS: M/Z=287 **(**M+H)$^{-}$

The IR, NMR and MS data are consistent with the structure shown in Figure 5.61 (1, 3′, 6, 7, tetrahydroxyisoflavone).

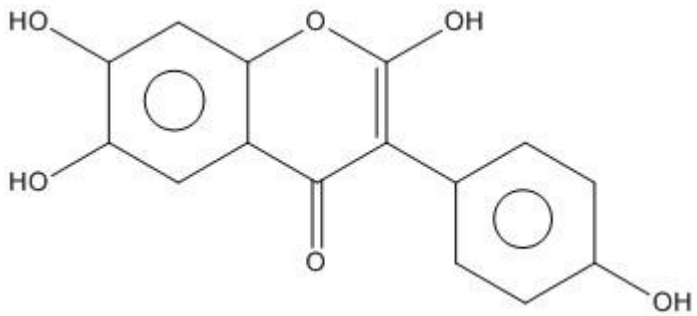


Figure 5.61: The proposed chemical structure of compound Es1 F3/3 ($C_{15}H_{10}O_6$, MW MZ=287 (M-H)$^{-}$).

Stack plots of ^{1}H NMR spectra from F6 and F3/3 showed very similar spectra (Figure 5.62).

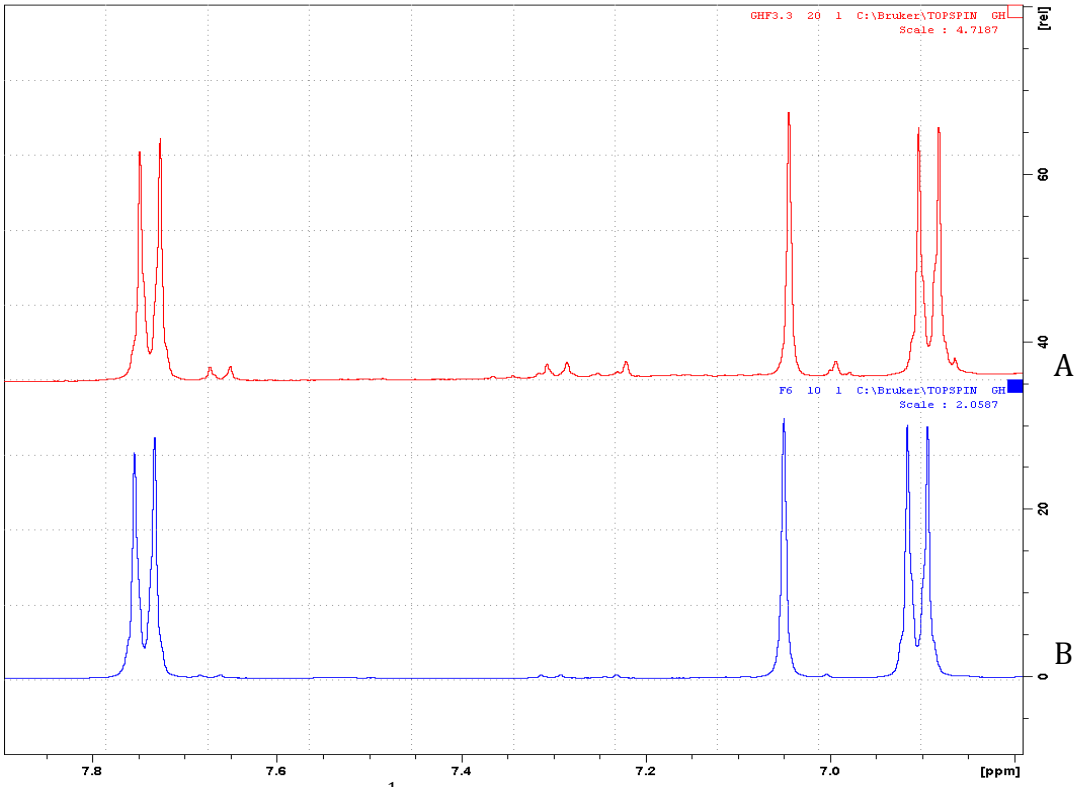


Figure 5.62: Comparisons of ^{1}H NMR spectra for Es1 F3.3 (A) and Es1 F6 (B)

Es1 Fraction 5

The NMR results of Es1 Fraction 5 are shown in Figures 5.63 to 5.68, Appendix 3.28 to 3.45. The ^{1}H NMR Spectrum was very similar to the one obtained from F3/1 (Figure 5.63) but this fraction had less impurities. The ^{1}HNMR spectrum and 2D COSY showed peaks at 7.73ppm (2H), 7.39pp (2H) and 7.14ppm (1H), 7.01ppm (1H), 6.93 ppm (1H), 6.9ppm (1H), 6.8ppm (1H) showing presence of two benzene rings, 1,4-disubstituted and 1,2,4-trisubstituted respectively (Figure 5.63 and 5.64). The ^{13}C NMR had fourteen signals, the signals at 175.03ppm, 174.85ppm corresponding to a carboxyl group and that at 161.32ppm with an enol group, the elven remaining signals 149.78ppm, 147.04ppm, 133.26ppm, 129.19ppm, 128.80ppm, 125.78ppm, 125.32ppm, 125.06ppm, 117.53ppm, 117.19ppm, 116.88 all fall into the aromatic region Figure 5.67. The JMOD ^{13}C NMR (Figure 5.68), 2 D g-HMQC (Figure 5.65) and 2 D g- HMBC spectra (Figure 5.66) were determined to aid structure determination. The FTIR spectrum is shown in Figure 5.69 and showed the same groups as F3/1. The range of MS showed too many signal on positive and negative mode (Appendix 3.46 to 3.48) and the MS of the compound showed a peak at 301 which corresponds to the $[M+H]^{-}$ ion in negative mode (Figure 5.70). A similar MS result was obtained from LC MS at University of Salford (Appendix 3.49).

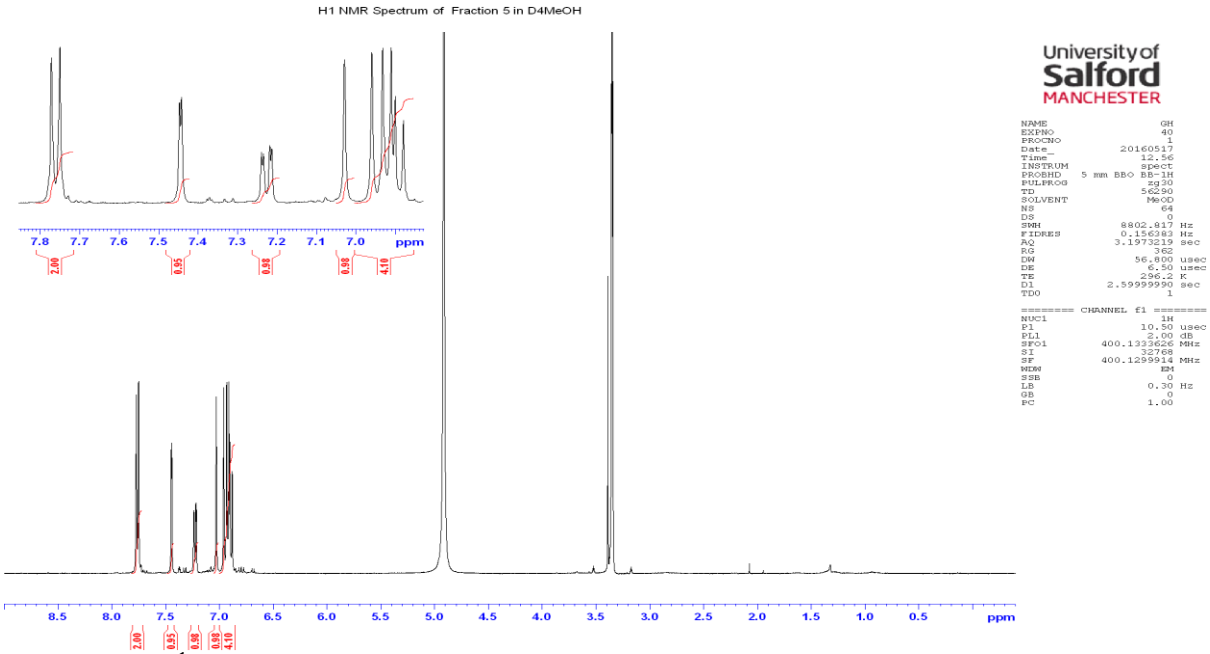


Figure 5.63: ^{1}H NMR Spectrum of Es1 F5 in CD_3OD+TMS

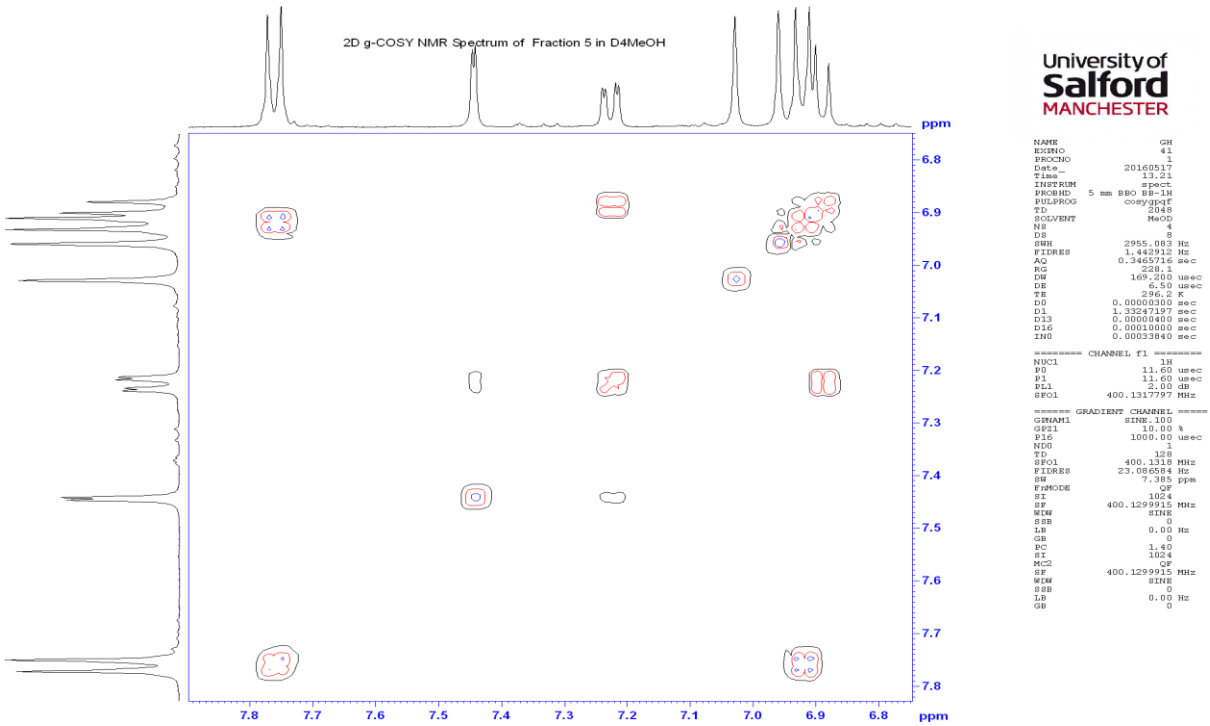


Figure 6.64: 2D g-COSY NMR Spectrum of Es1 F5 (expansion) in CD_3OD

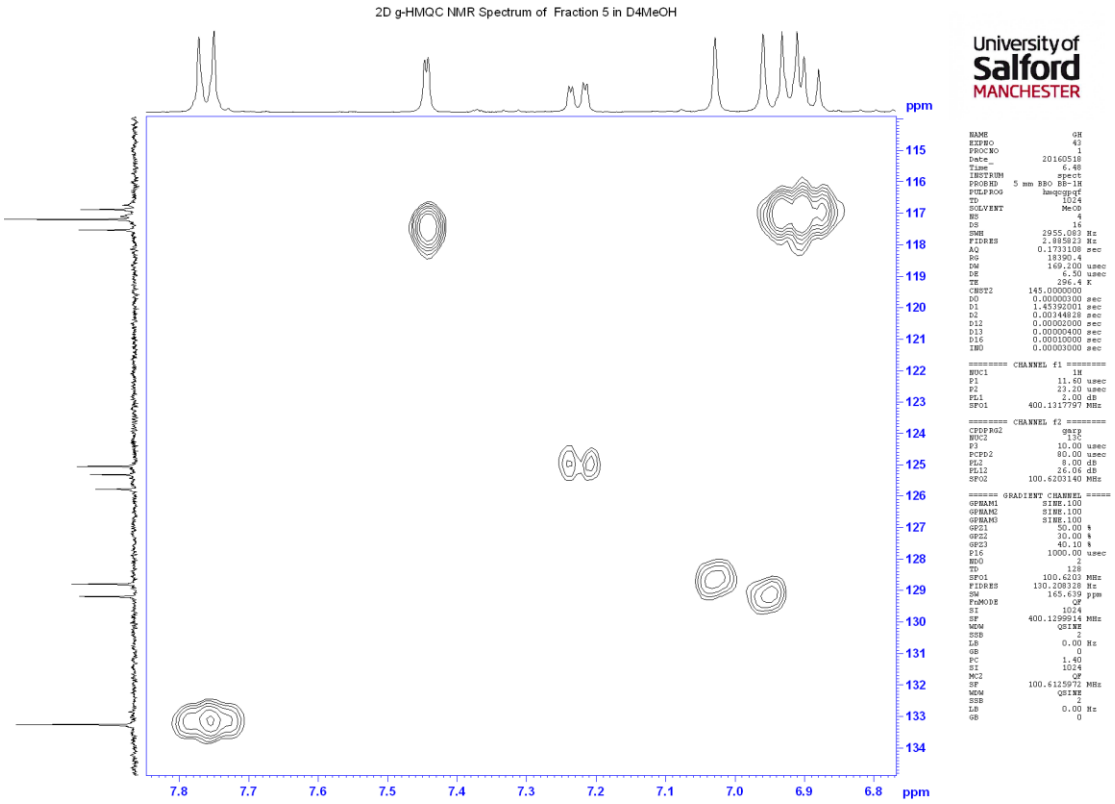


Figure 5.65: 2D g-HMQC Spectrum of Es1 F5 (expansion) in CD_3OD

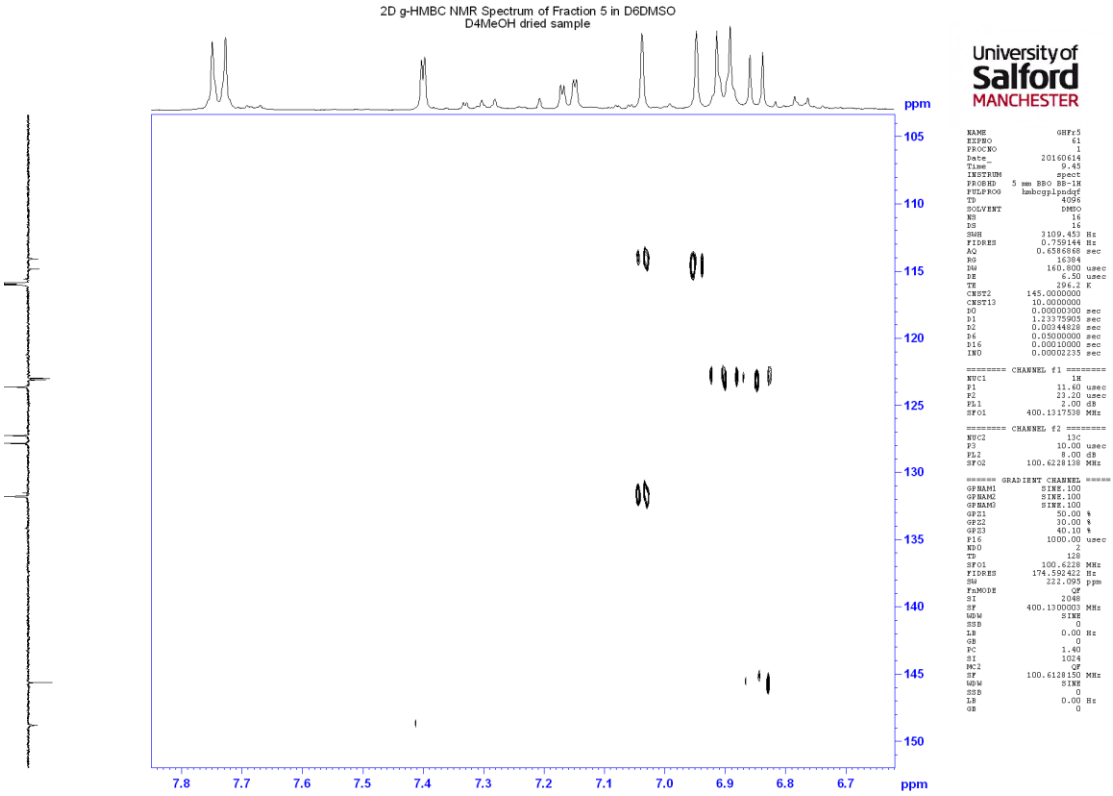


Figure 5.66: 2D g-HMBC Spectrum of Es1 F5 (expansion) in CD_3OD

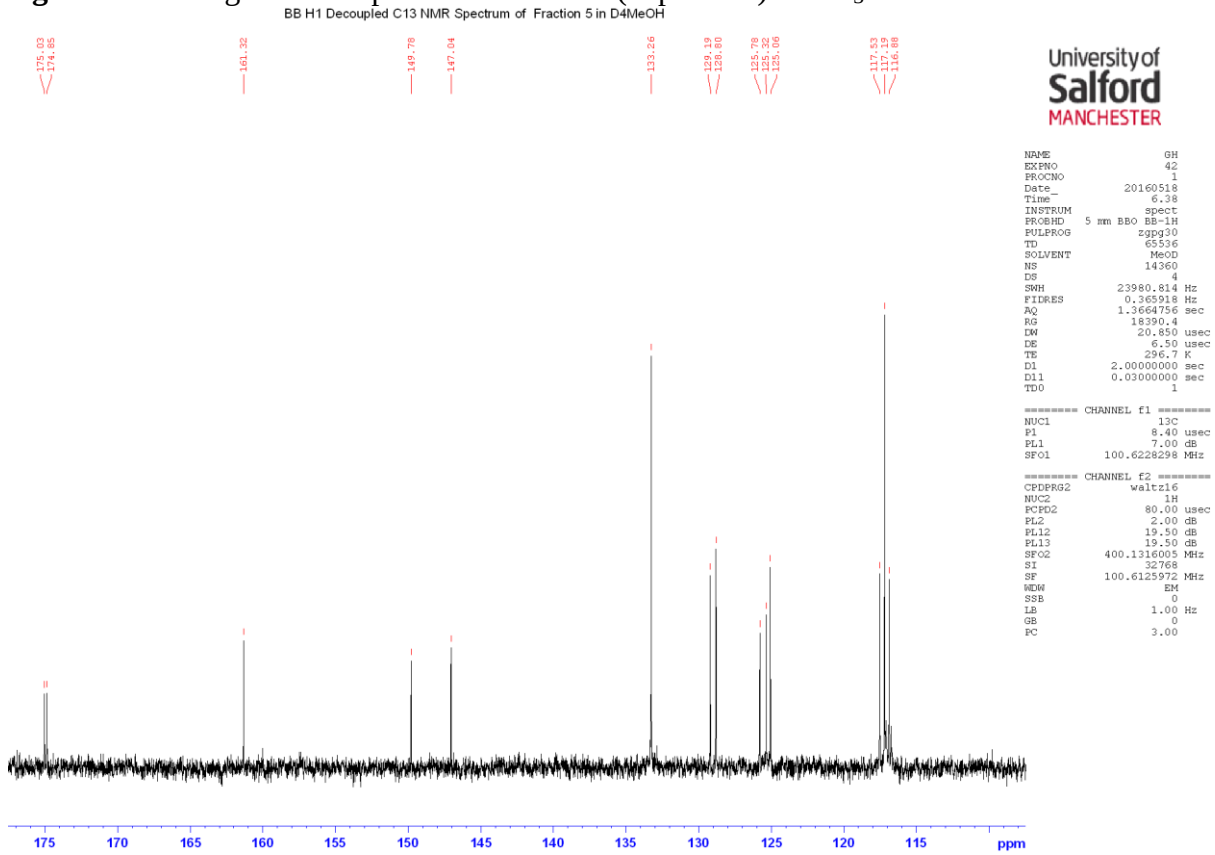


Figure 5.67: BB H1 Decoupled C13 NMR Spectrum of Es1 F5 (expansion) in CD_3OD

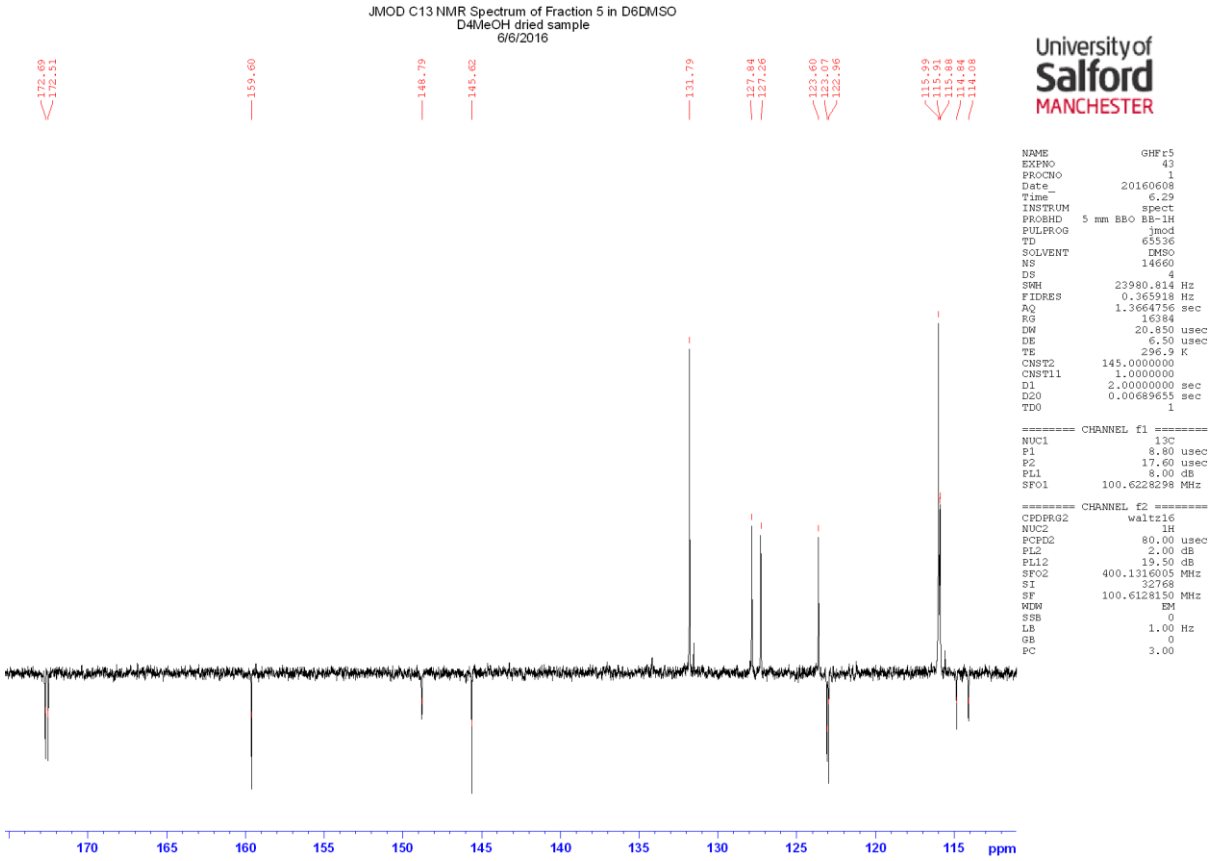


Figure 5.68: JMOD C13 Spectrum of Es1 F5 (expansion) in CD_3OD

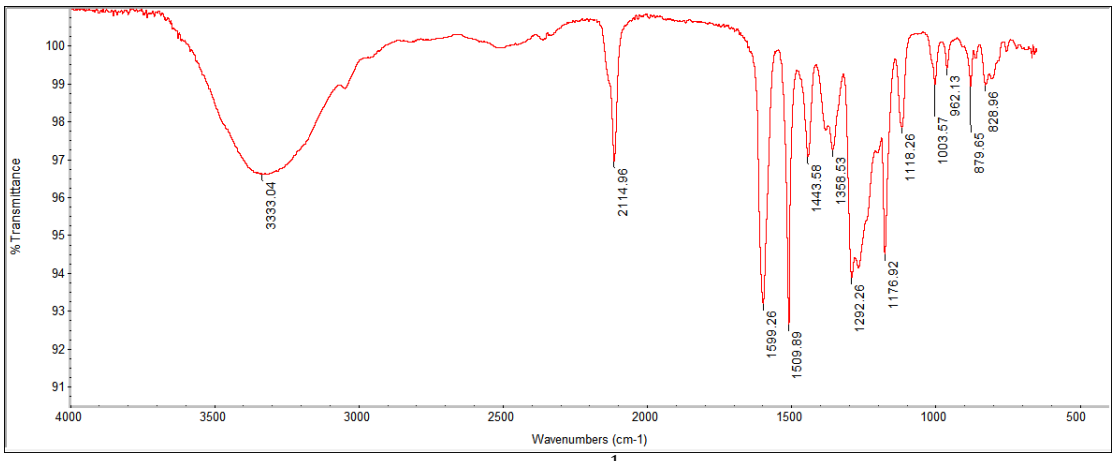


Figure 5.69: The FTIR spectrum (ATR) 4 cm $^{-1}$ (16 Scans) of Es1 F5

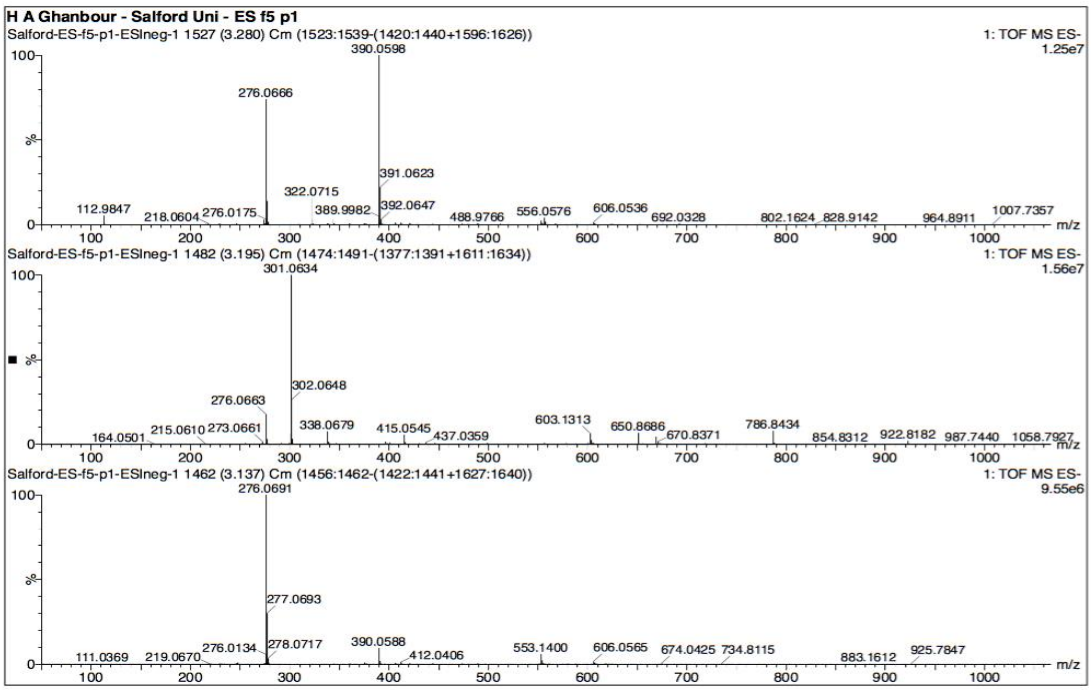


Figure 5.70: The negative ion spectrum from LC MS-TOF of Es1 F5

Summary of spectral data for compound of Es1 F 5

^{1} H-NMR (400 MHz, CD_3OD): δ H 7.73(d of d), 7.39(d), 7.14(d of d), 7.01(s), 6.93(s), 6.9(d of d) and 6.8(d)

^{13}C NMR (400 MHz, CD_3OD): δ ^{13}C 175.03, 174.85, 161.32, 149.78, 147.04, 133.26, 129.19, 128.80, 125.78, 125.32, 125.06, 117.53, 117.19 and 116.88

FTIR spectra (ATR) 4 cm $^{-1}$ (16 Scans): 3333 (br), 2114 (s), 1599 (s), 1509 (s), 1443 (ss), 1358 (ss), 1292 (br), 1176 (s), 1118 (ss), 1003 (ss), 962 (ss), 879 (ss) and 828 (sbr)

MS: MZ=301 **(**M-H)$^{-}$

The IR, NMR and MS data are consistent with the structure shown in Figure 5.71 (2, 2', 4′, 6, 8, pentahydroxyisoflavone).

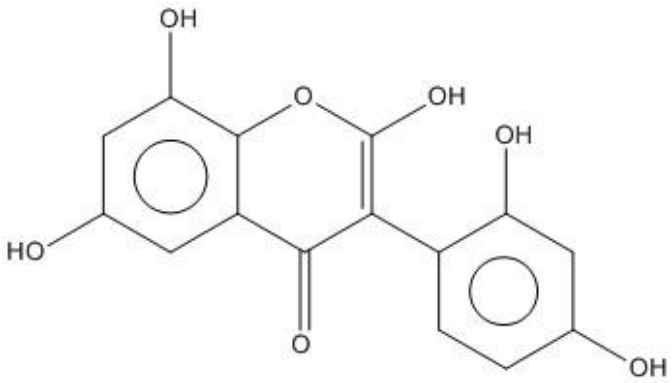


Figure 5.71: The confirmed chemical structure of compound Es1 F5 ($C_{15}H_{10}O_7$, MW MZ=301 (M-H)-).

Stack plots of ^{1}H NMR spectra from Es1 F5 and Es1 F3/1 showed very similar spectra with different shifts on some peaks (arrows Figure 7.72).

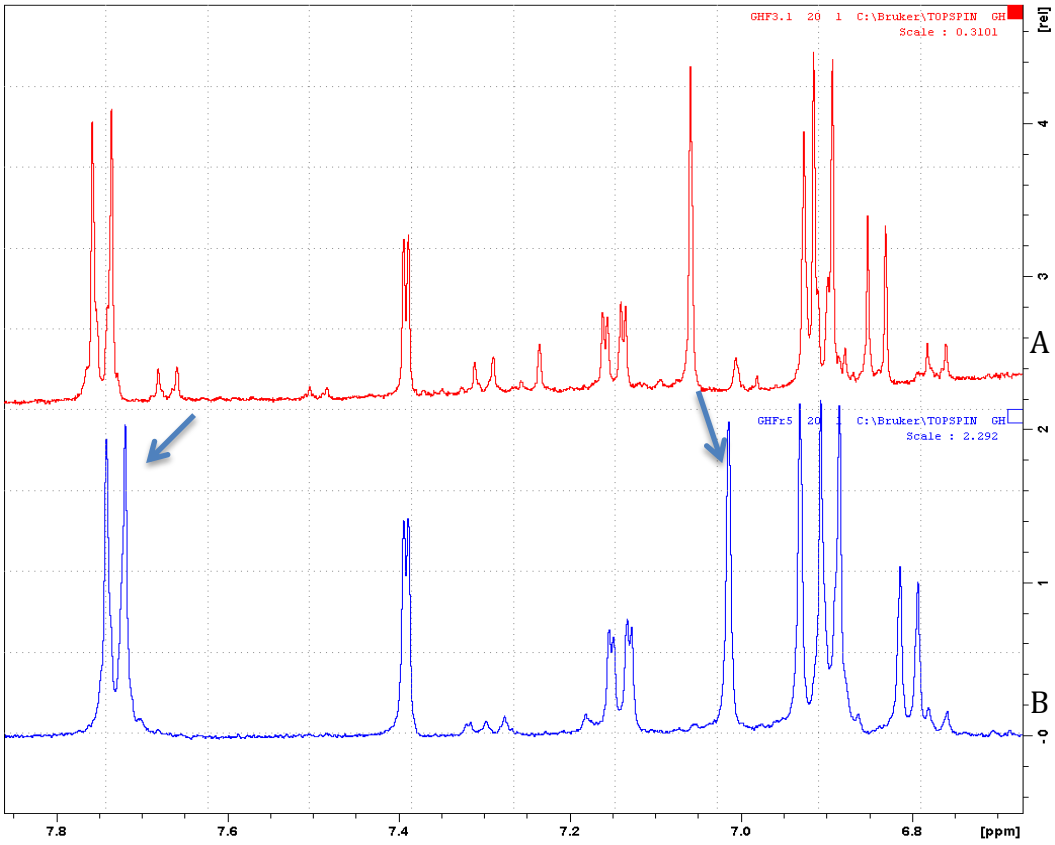


Figure 5.72: Comparison of ^{1}H NMR spectra for Es1 F3/1 (A) and F5 (B)

5.2.2.2 M1 11 Fractions

NMR: Spectroscopy and Interpretation

M1 Fraction1 2 p4

The NMR results of M1 F1 2 p4 are shown in Figures 5.73 to 5.74, Appendix 3.50 to 3.52. The NMR data showed the sample to be impure (Figure 5.73), As a result the sample was purified by HPLC to obtain a single compound purified compound from M1 F1 2 p4. ^{1}H NMR Spectrum showed five protons in aromatic rings (Figure 5.74). The ^{13} C spectrum was too weak due to lack of material and did not show all signals (Appendix 3.52). The FTIR shown in (Figure 5.76) and showed alcohol group (3416 cm $^{-1}$), aromatic group (2323 cm $^{-1}$) and alkene group (2161 cm $^{-1}$). The range of MS showed too many signal on positive and negative mode (Appendix 3.53 to 3.55) and the MS of the compound showed a peak at 285 which corresponds to the $[M+H]^{-}$ ion in negative mode (Figure 5.77).

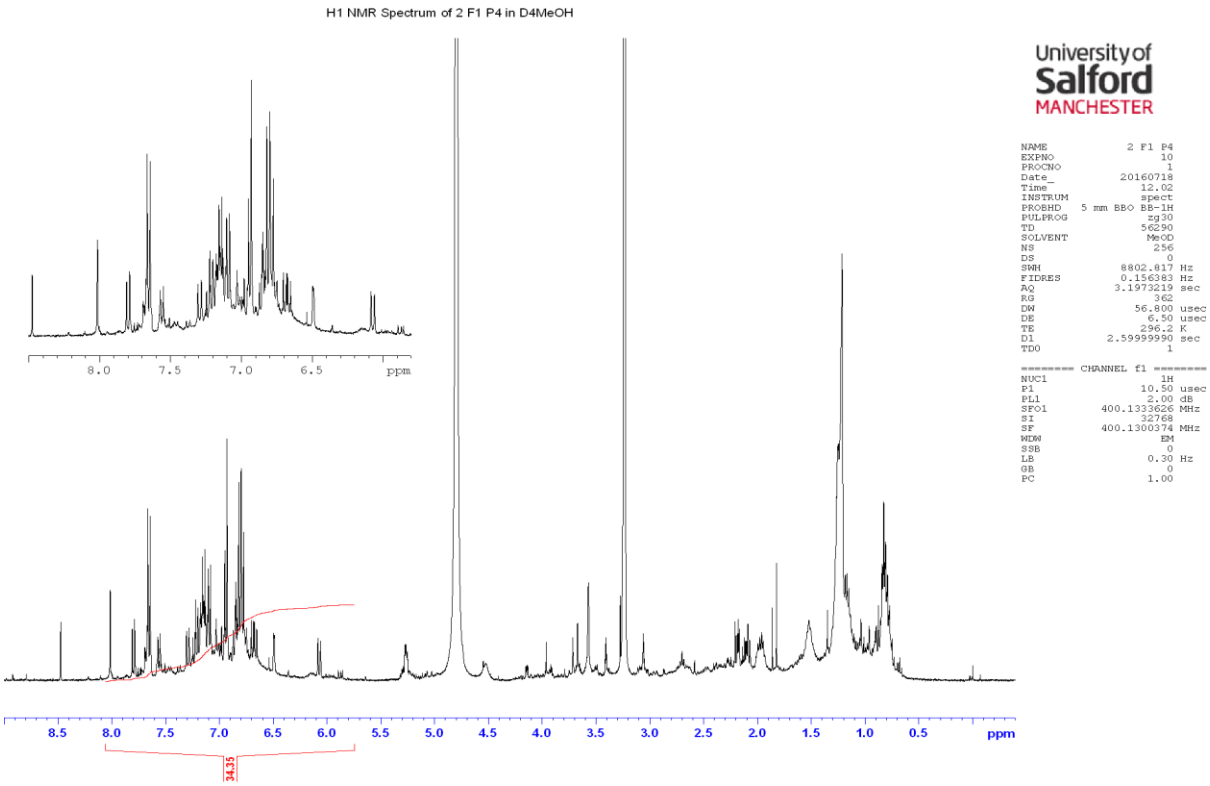


Figure 5.73: ^{1}H NMR Spectrum of M1 F1 2 P4 in CD_3OD+TMS

The sample was too impure so the spectra obtained were too complicated and was not possible to interpret. The sample was purified and only <1.8 mg was obtained therefore only ^{1}H and COSY experiments were performed and as a result the structure was not confirmed.

Purified compound from M1 F1 2 p4

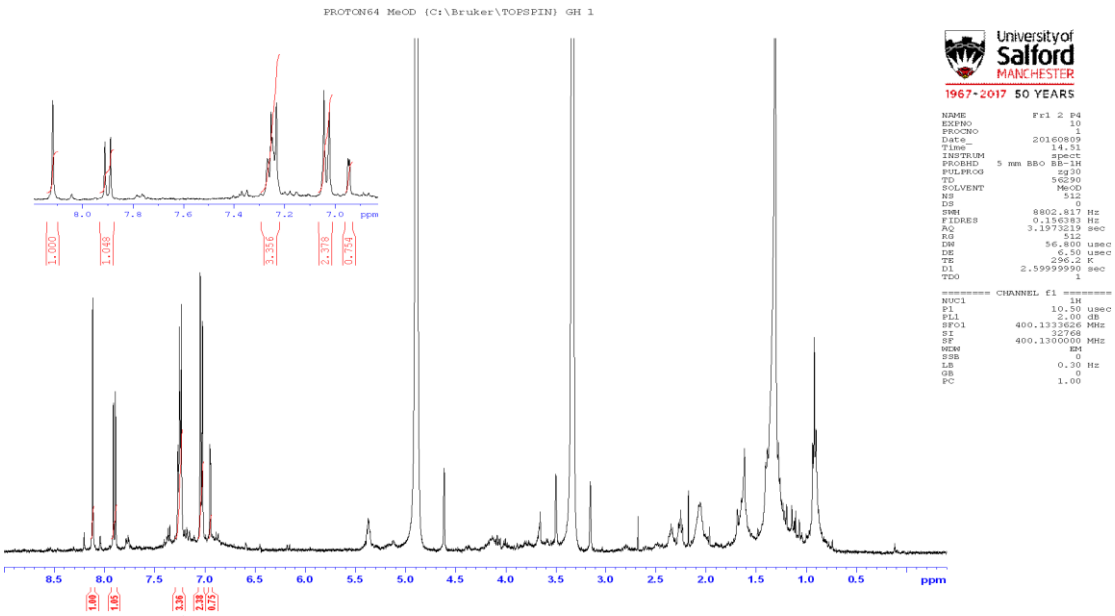


Figure 5.74: ^{1}H NMR Spectrum of M1 F1 2 P4 in CD_3OD+TMS

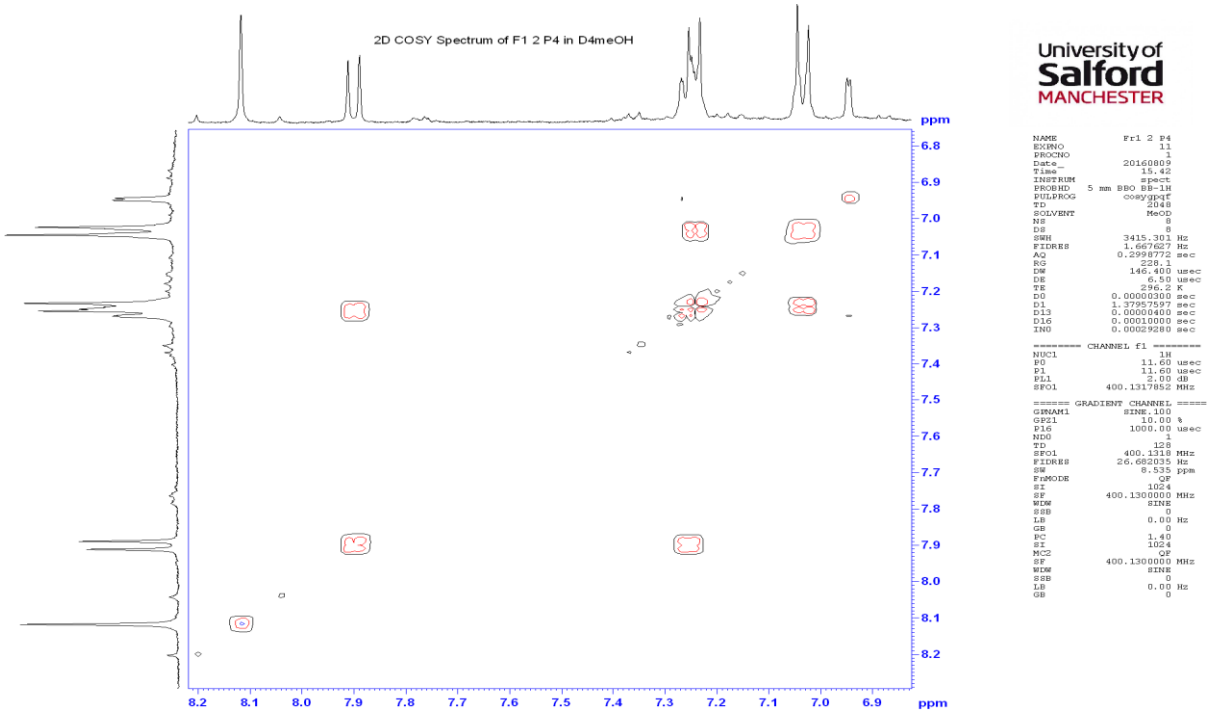


Figure 5.75: 2D g-COSY NMR Spectrum of M1 F1 2 P4 in CD_3OD acquired with smaller sweep width (SW) manually due a sample limited amount

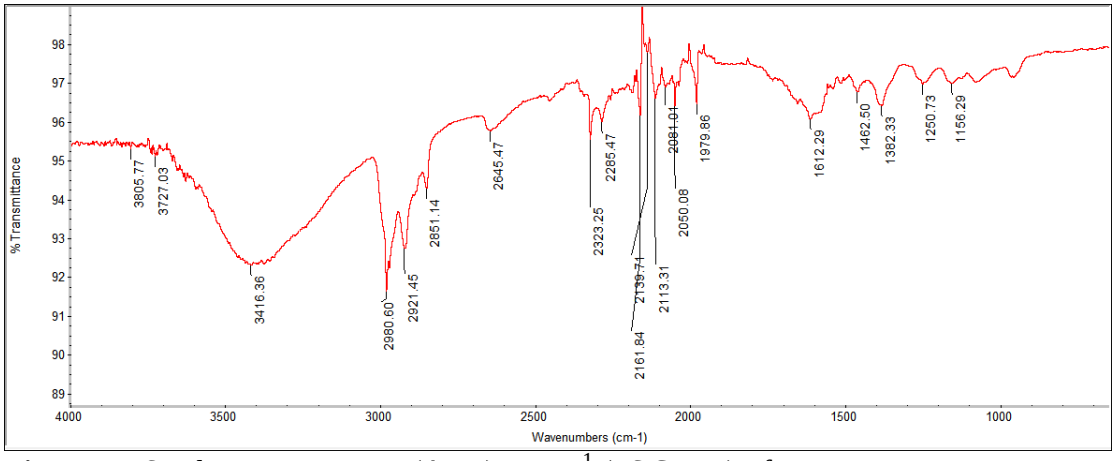


Figure 5.76: The FTIR spectra (ATR) 4 cm $^{-1}$ (16 Scans) of M1 F1 2 p4

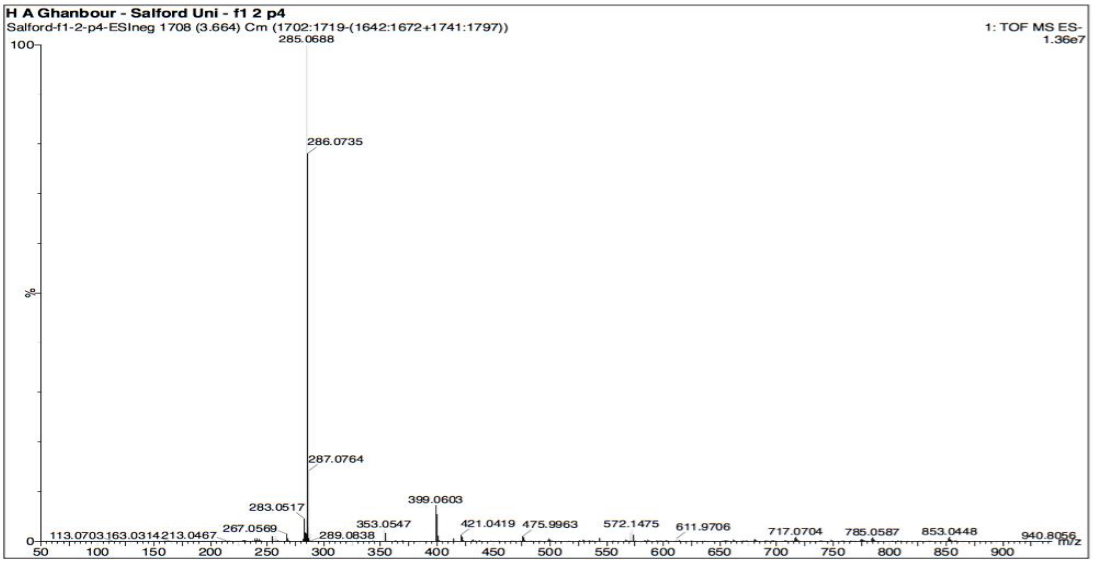


Figure 5.77: The negative ion spectrum from LC MS-TOF of M1 F1 2 p4

Summary of spectral data of compound M1 **F1 2 p4**

^{1}H-NMR (400 MHz, CD_3OD): δ H 8.12 s, 7.9 (d), 7.26 (d of d), 7.24 (d of d), 7.03 (d of d) and 6.95 (d)

^{13}C NMR (400 MHz, CD_3OD):

Due limited amount of material ^{13}C NMR was not obtained

FTIR spectra (ATR) 4 cm $^{-1}$ (16 Scans): 3367(br), 2980(ms), 2923(s), 2852(ss), 2116(s), 1612(s), 1514(ms), 1449(sbr), 1385(sbr), 1238(br) and 1172(ss)

MS: M/Z= 285 **(**M-H)$^{-}$

Six fractions (M1 F1 1 p3, M1 F1 3 p5, M1 F1 4 p6, M1 F2 2 p2, M1 F3 1 p2 and M1 F3 2 p3) with very similar data to M1 F1 2 p4 are shown in the Appendix (3.56 to 3.103)

M1 Fraction 3 3 p4

The NMR results of M1 F3 3 p4 are shown in Figures 5.78 to 5.80, Appendix 3.104 to 3.109. Data was not clear enough to define the confirmed structure but there was a clear signal on alkene proton (6.8 ppm) stronger than in the aromatic region (Figure 5.78). The ^{1}H NMR and 2D COSY spectra showed peak at 6.78ppm (2H), 7.63pp (2H) and 6.9ppm (1H), showing presence of possibly two benzene rings, 1,4-disubstituted and penta substituted benzene ring respectively or an olefinic proton (Figure 5.78-7.80). Signal from ^{13} C, g-HMQC and HMBC showed the sample to be impure (Appendix 3.105, 3.107 and 3.109). The fraction was run on a better NMR instrument at Cambridge University to get higher resolution data but the results were the same as the Salford University results (Figure 5.80 and Appendix 3.105 to 3.109). The FTIR spectrum is shown in Figure 5.81 and showed the same groups as M1 F1 2 p4. The range of MS showed to many signal on positive and negative mode (Appendix 3.110 to 3.112) and the MS of the compound showed a peak at 287 which corresponds to the $[M+H]^{-}$ ion in negative mode (Figure 5.82).

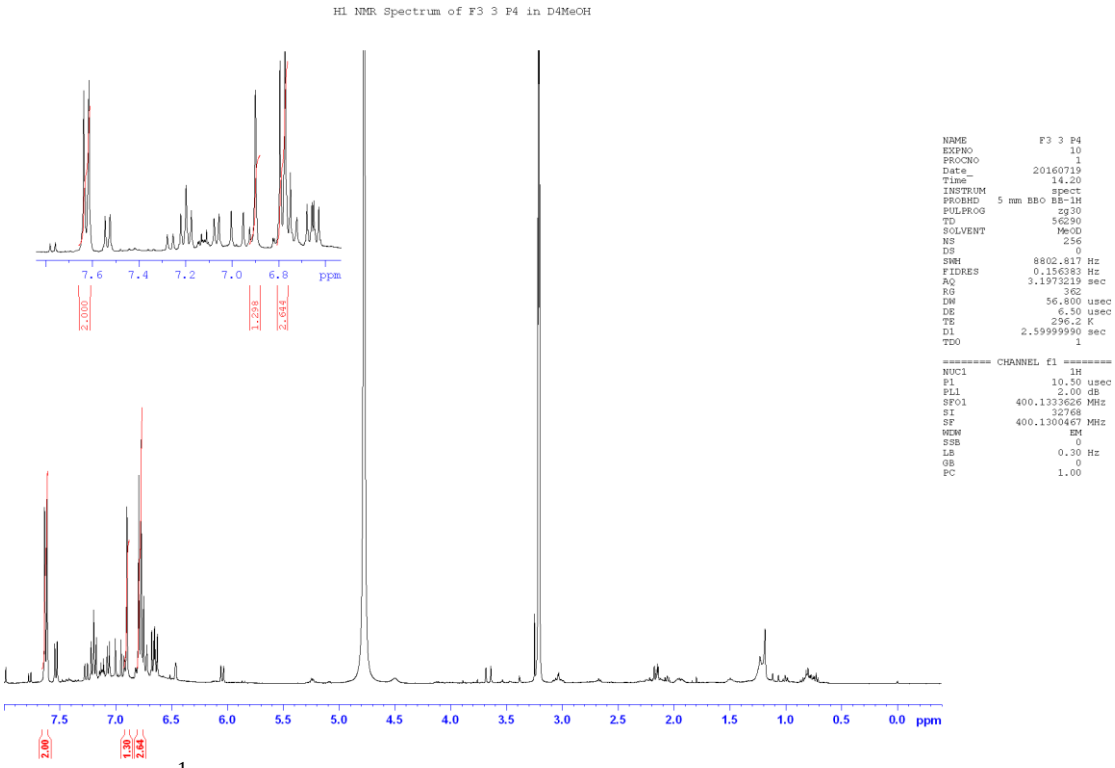


Figure 5.78: ^{1}H NMR Spectrum of M1 F3 3 p4 in CD_3OD+TMS

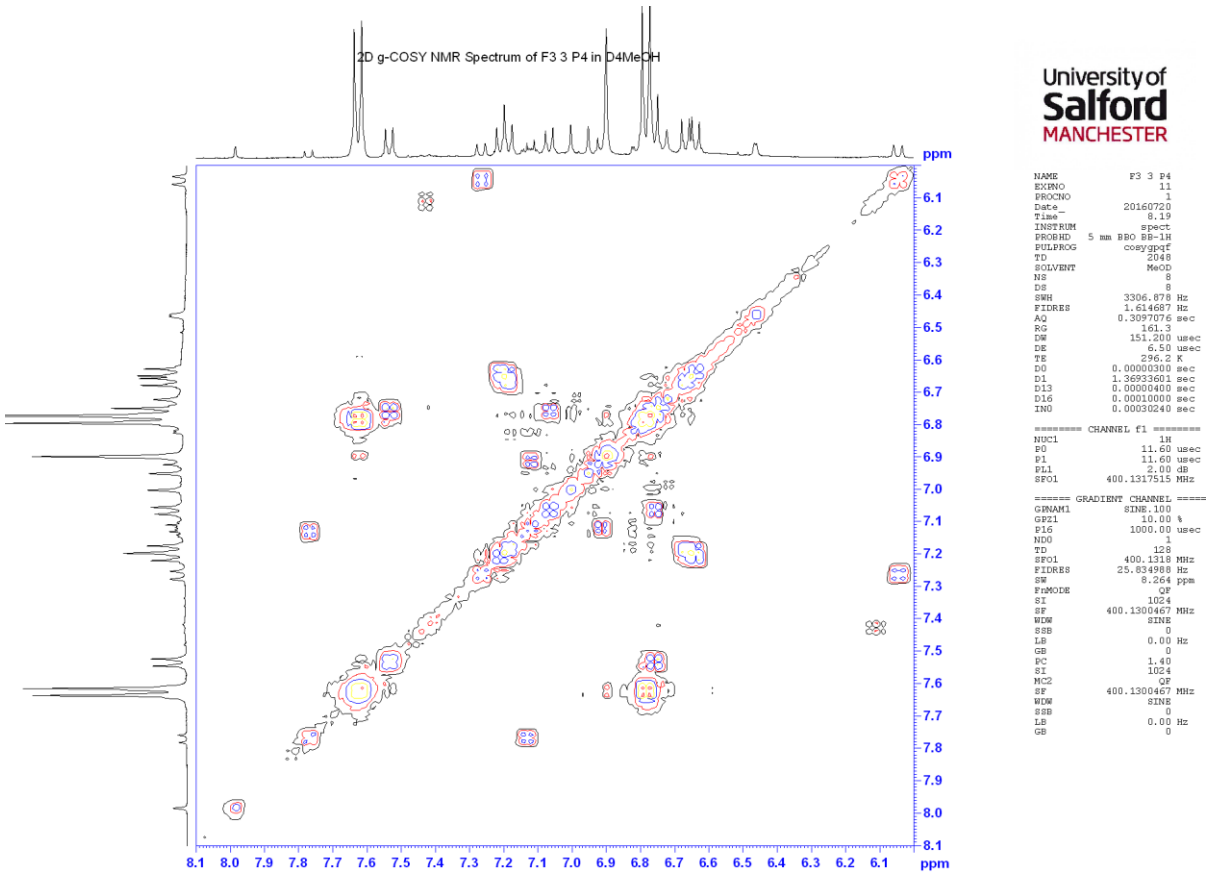


Figure 5.79: 2D g-COSY NMR Spectrum of M1 F3 3 p4 (expansion) in CD_3OD

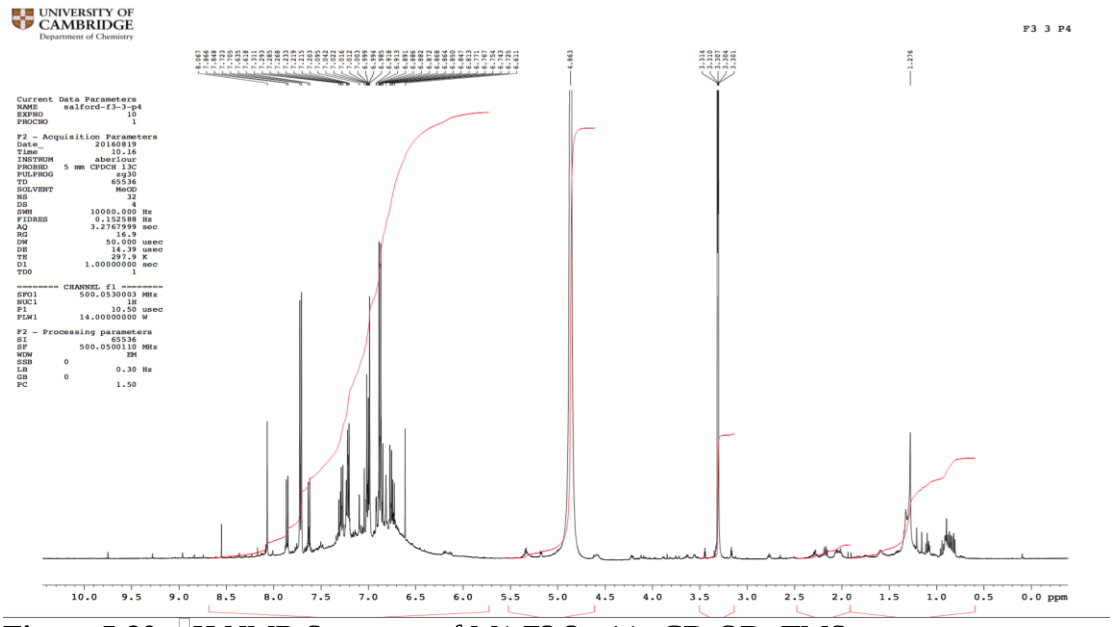


Figure 5.80: ^{1}H NMR Spectrum of M1 F3 3 p4 in CD_3OD+TMS

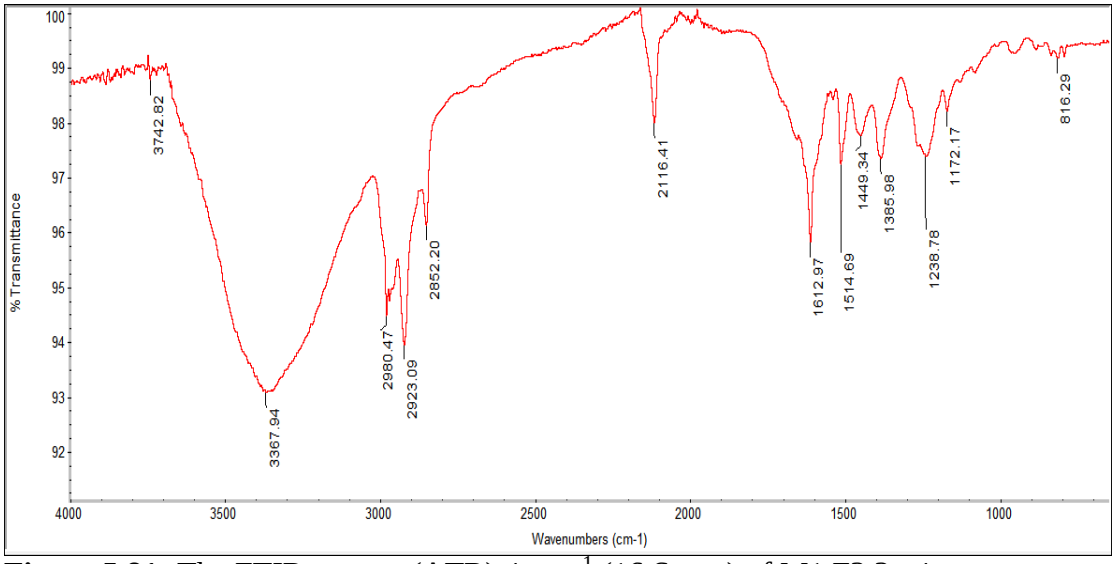


Figure 5.81: The FTIR spectra (ATR) 4 cm $^{-1}$ (16 Scans) of M1 F3 3 p4

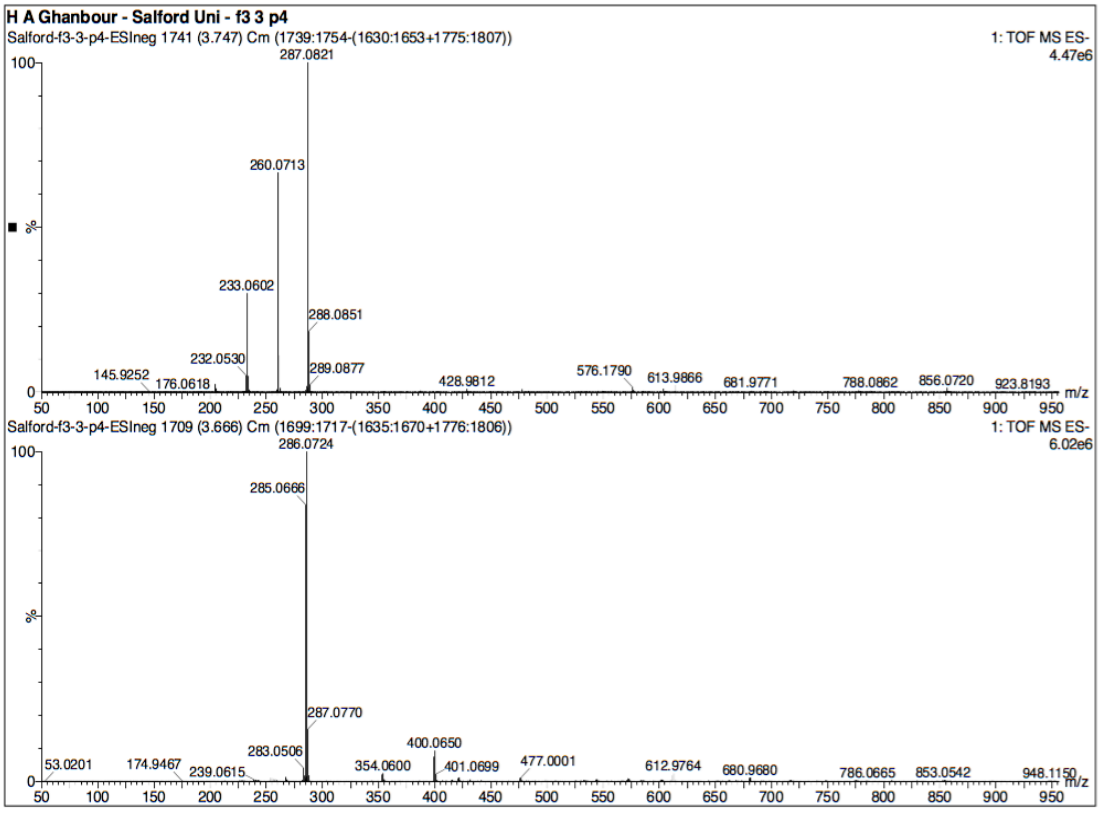


Figure 5.82: The negative ion spectrum from LC MS-TOF of M1 F3 3 p4

Summary of spectral data of compound M1 F3 3 p4

^{1}H-NMR (400 MHz, CD_3OD): δ H 7.63 (d of d), 6.90 (s), 6.78 (d of d)

^{13}C NMR (400 MHz, CD_3OD): δ ^{13}C 174.61, 161.07, 135.60, 135.33, 133.05, 132.99, 132.57, 132.39, 132.12, 131.46, 128.65, 127.52, 124.98, 122.52, 116.92, 116.76 and 116.64

FTIR spectra (ATR) 4 cm $^{-1}$ (16 Scans): 3367(br), 2980(ms), 2923(s), 2852(ss), 2116(s), 1612(s), 1514(ss), 1449(sbr), 1385(sms) and 1238(br)

MS: M/Z= 287 **(**M+H)$^{-}$

The FTIR, NMR and MS data are consistent with the structure shown in Figure 5.83 (1′, 3′, 4′, 8 tetrahydroxyflavone).

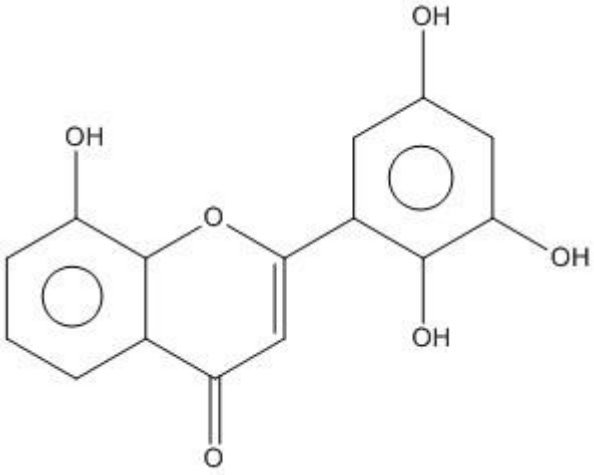


Figure 5.83: The chemical structure of compound M1 F3 3 p4 ($C_{15}H_{10}O_6$, MW MZ=287 (M+H)-).

M1 Fraction 3 4 p5

The NMR results of M1 F3 4 p5 are shown in Figures 5.84 to 5.89, Appendix 3.113 to 3.117. The ^{1}H NMR spectrum showed good signal in the aromatic region, five proton aromatic type and one proton alkene type (Figure 5.84). The 2 D g-Cosy showed two aromatic rings, the ^{1}H NMR spectrum and 2D COSY spectrum showed peak at 7.11ppm (2H), 6.91ppm (2H) and 7.76ppm (1H), 7.14 ppm (1H), 6.82ppm (1H), 7.98ppm (1H), showing the presence of two benzene rings, 1,4-disubstituted and 1,2,4-trisubstituted respectively and an olefinic proton (Figure 5.84 and 5.85). The ^{13}C NMR had fifteen signals, the signals at 172.6ppm, 170.3ppm corresponding to a carboxyl group and that at 160.4ppm, 159.7ppm with an enol group. The eleven remaining signals 139.5ppm, 135.8ppm, 132.4ppm, 131.8ppm, 128.3ppm, 127.8ppm, 127ppm, 122.9ppm, 118.7ppm, 117.1ppm, 110.2 all fall into the aromatic region (Figure 5.86), the JMOD ^{13}C NMR (Figure 5.89), 2 D g-HMQC (Figure 5.87) and 2 D g- HMBC spectra (Figure 5.88) were determined to aid structure determination. The FTIR spectrum is shown in Figure 5.90 and showed the same groups asM1 F1 2 p4. The range of MS showed to many signal on positive and negative mode (Appendix 3.118 to 3.120) and the MS of the compound showed a peak at 285 which corresponds to the $[M+H]^-$ ion in negative mode (Figure 5.91) and the same MS result was obtained from LC MS at Salford University (Appendix 3.121).

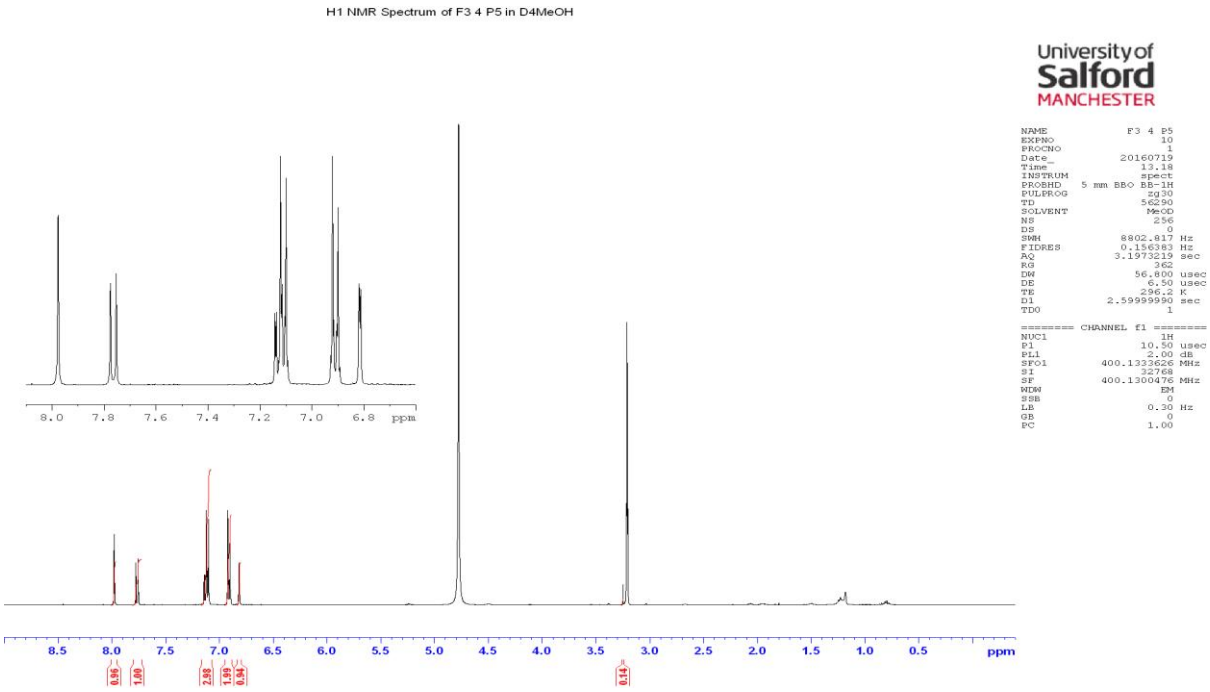


Figure 5.84: H NMR Spectrum of M1 F3 4 p5 in CD_3OD+TMS

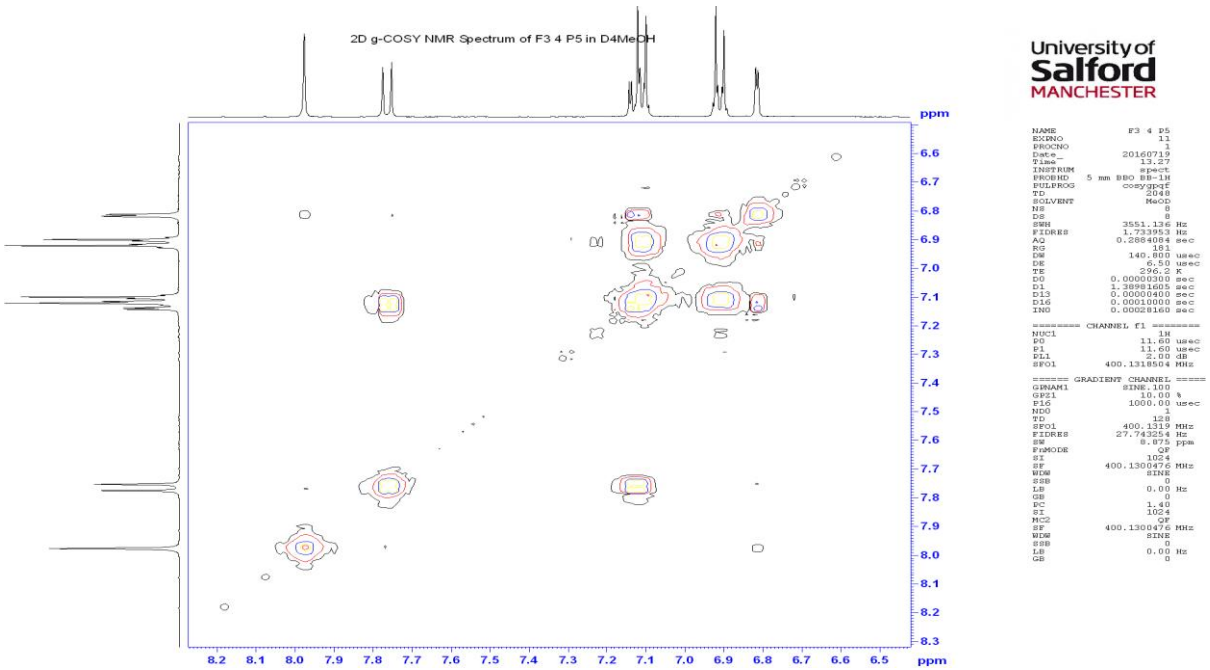


Figure 5.85: 2D g-COSY NMR Spectrum of M1 F3 4 p5 (expansion) in CD_3OD

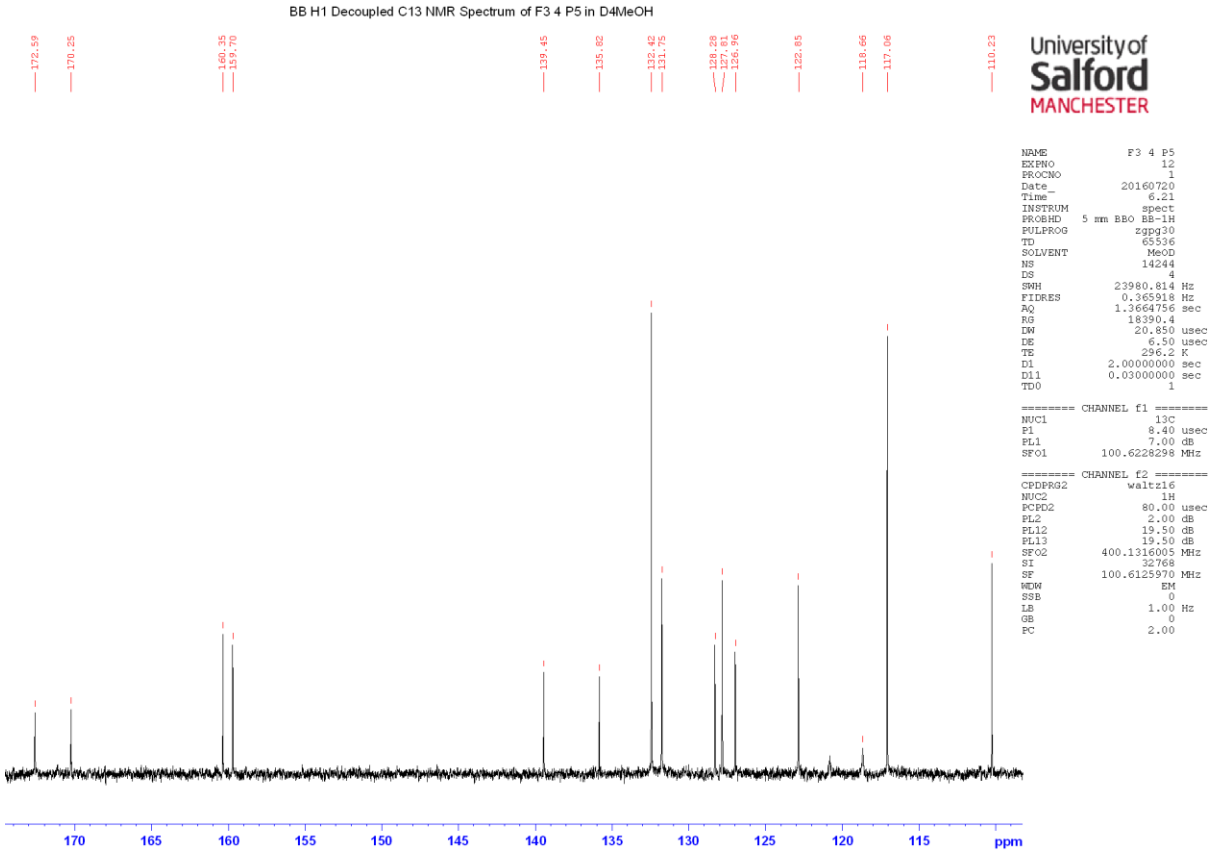


Figure 5.86: BB H1 Decoupled C13 Spectrum of M1 F3 4 p5 (expansion) in CD_3OD

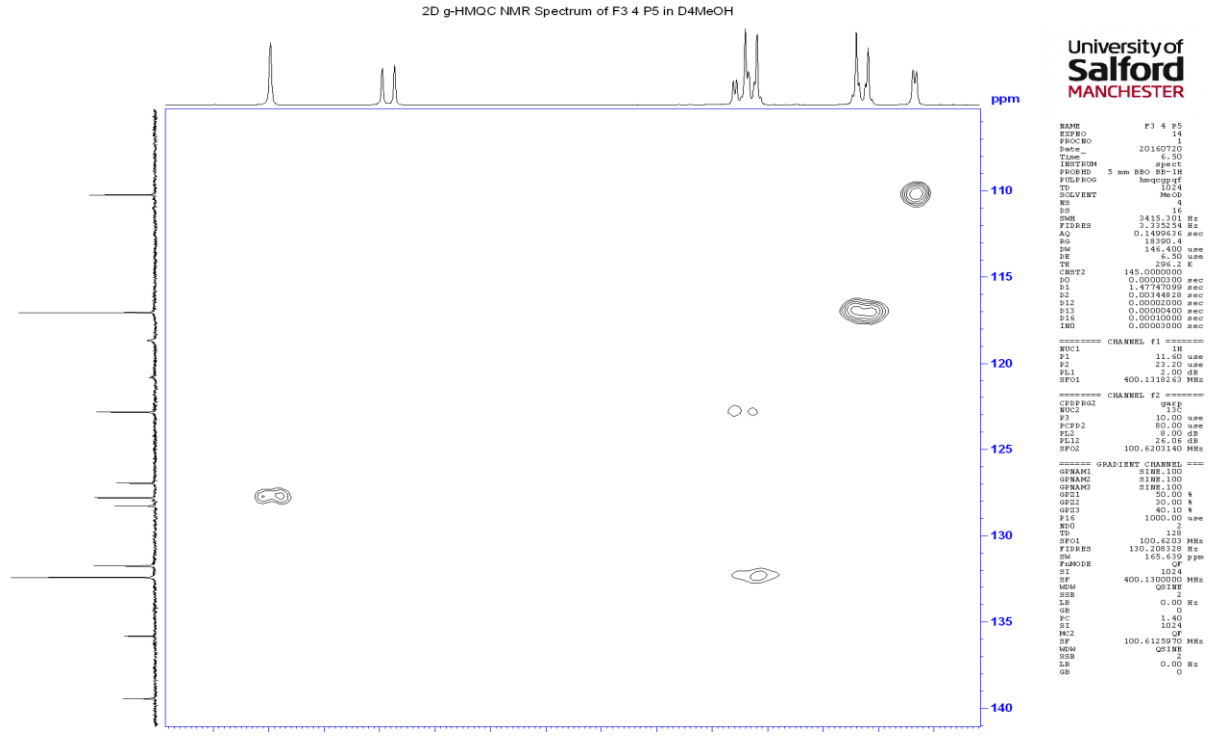


Figure 5.87: 2D g-HMQC Spectrum of M1 F3 4 p6 (expansion) in CD_3OD

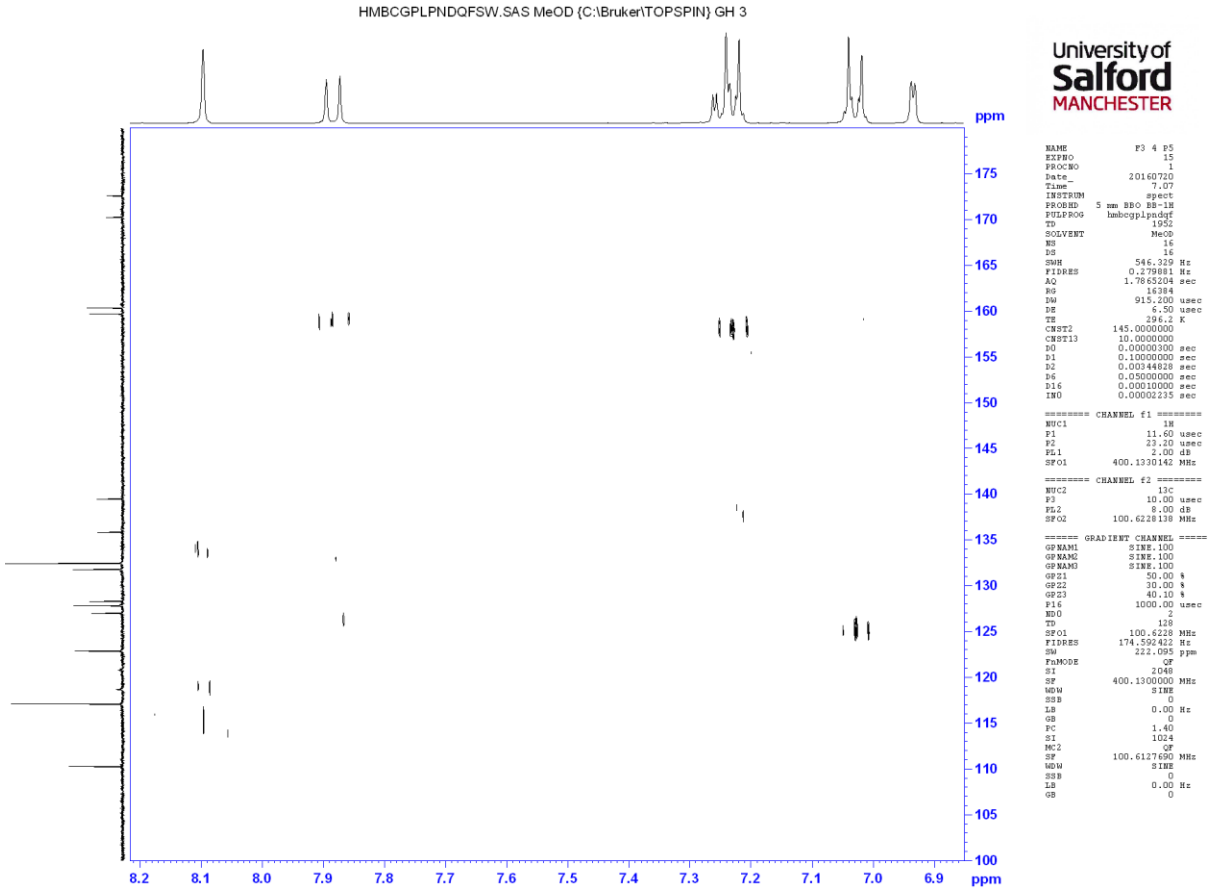


Figure 5.88: 2D g-HMBC NMR Spectrum of M1 F3 4 p5 (expansion) in CD_3OD

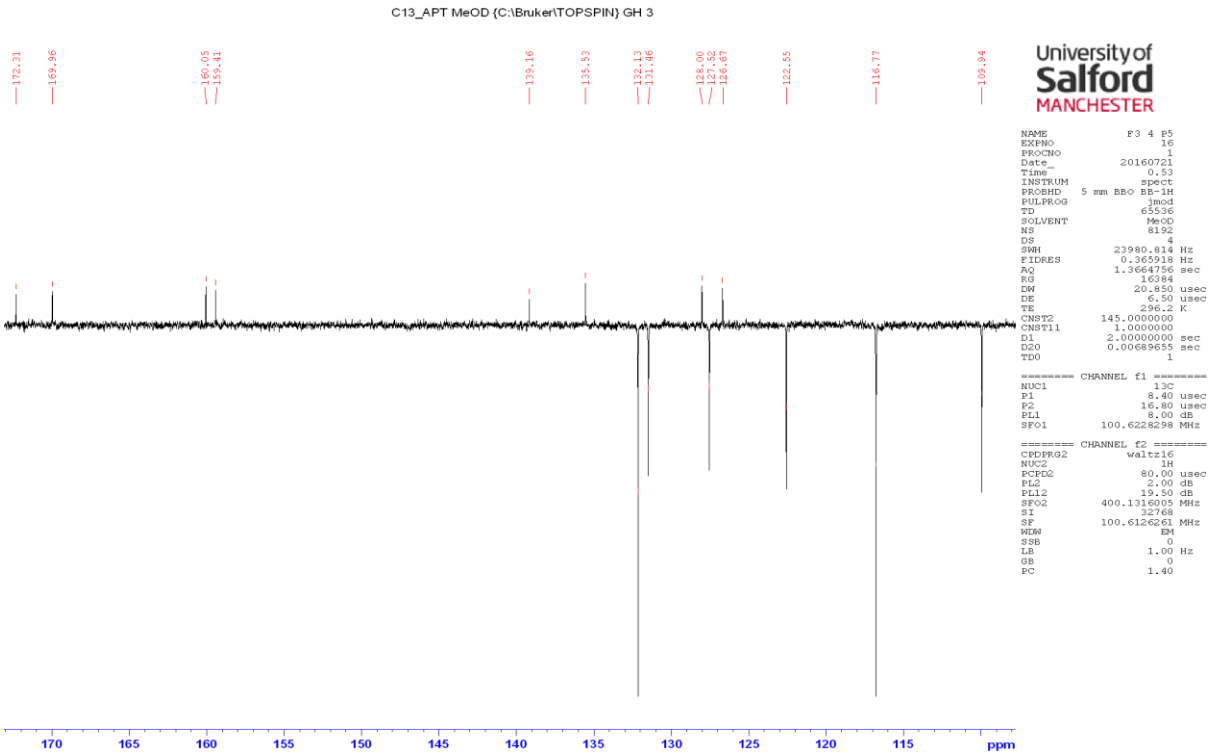


Figure 5.89: JMOD C13 Spectrum of M1 F3 4 p5 in (expansion) CD_3OD

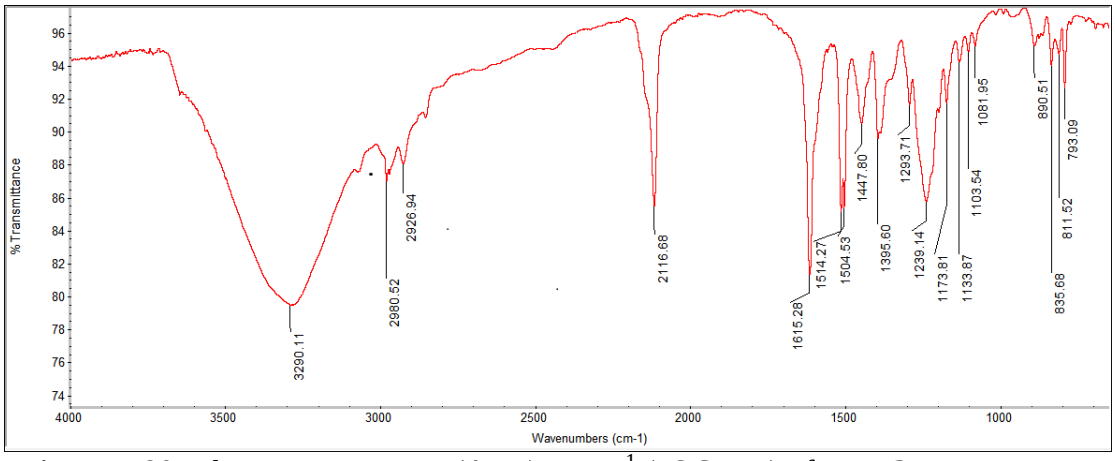


Figure 5.90: The FTIR spectrum (ATR) 4 cm $^{-1}$ (16 Scans) of M1 F3 4 p5

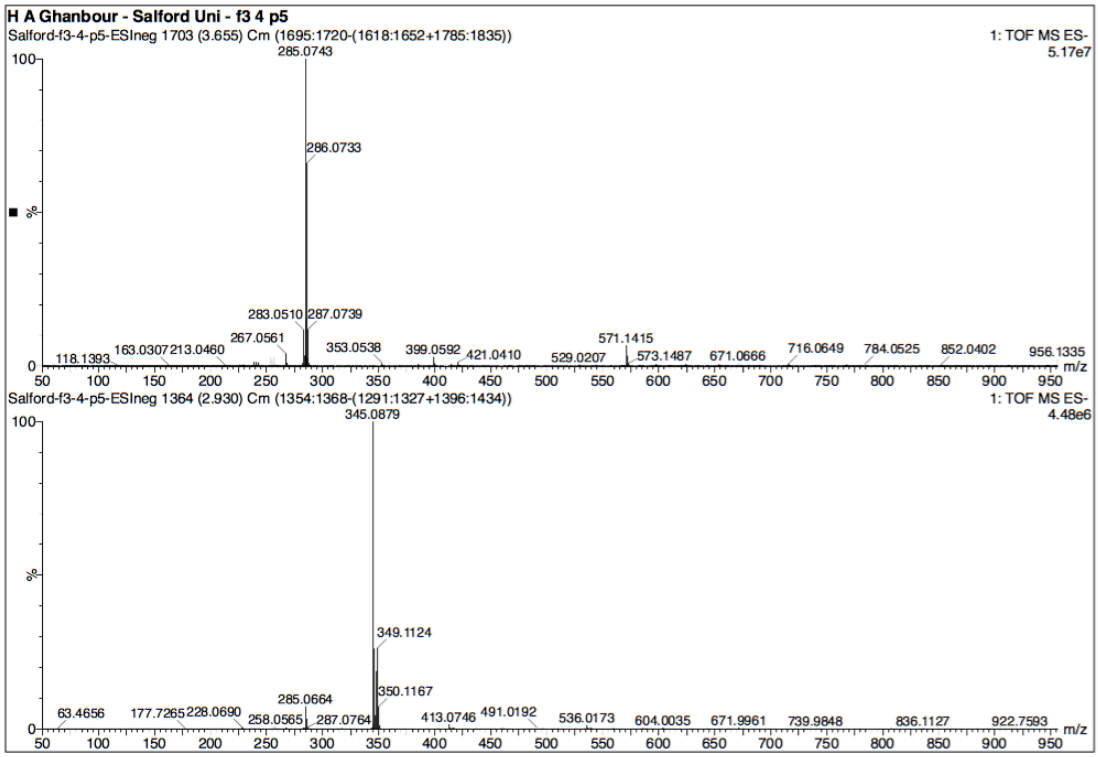


Figure 5.91: The negative ion spectrum from LC MS-TOF of M1 F3 4 p5

Summary of spectral data of compound M1 F3 4 p5

^{1}H-NMR (400 MHz, CD_3OD) ppm: δ H 7.98(s), 7.76(d), 7.14(d of d), 7.11(d of d), 6.91(d of d) and 6.82(d)

^{13}C NMR (400 MHz, CD_3OD): δC 172.6, 170.3, 160.4, 159.7,139.5, 135.8, 132.4, 131.8, 128.3, 127.8, 127, 122.9, 118.7, 117.1 and 110.2

FTIR spectra (ATR) 4 cm $^{-1}$ (16 Scans): 3290(br), 2980(sms), 2926(sms), 2116(s), 1615(s), 1504(ms), 1447(ms), 1395(ms) and 1239(ms),

MS: M/Z= 285 **(**M-H)$^{-}$

The IR, NMR and MS data are consistent with the structure shown in Figure 5.92 (2', 4', 5, 8 tetrahydroxyisoflavone).

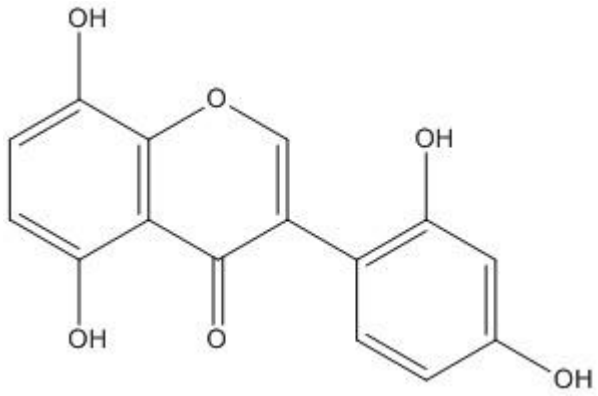


Figure 5.92: The confirmed chemical structure of compound F3 4 p5 ($C_{15}H_{10}O_6$, MW MZ=285 (M-H)).

Stack plots of ^{1}H NMR spectra from F3 4 p5, M1 F3 1 p2 and M1 F1 4 p6 gave similar spectra as did M1 F3 1 p2, M1 F2 2 p3, M1 F1 2 p4 and M1 F1 3 p5 with different shifts on some of the peaks (Figure 5.93) suggesting that the compounds had similar structures.

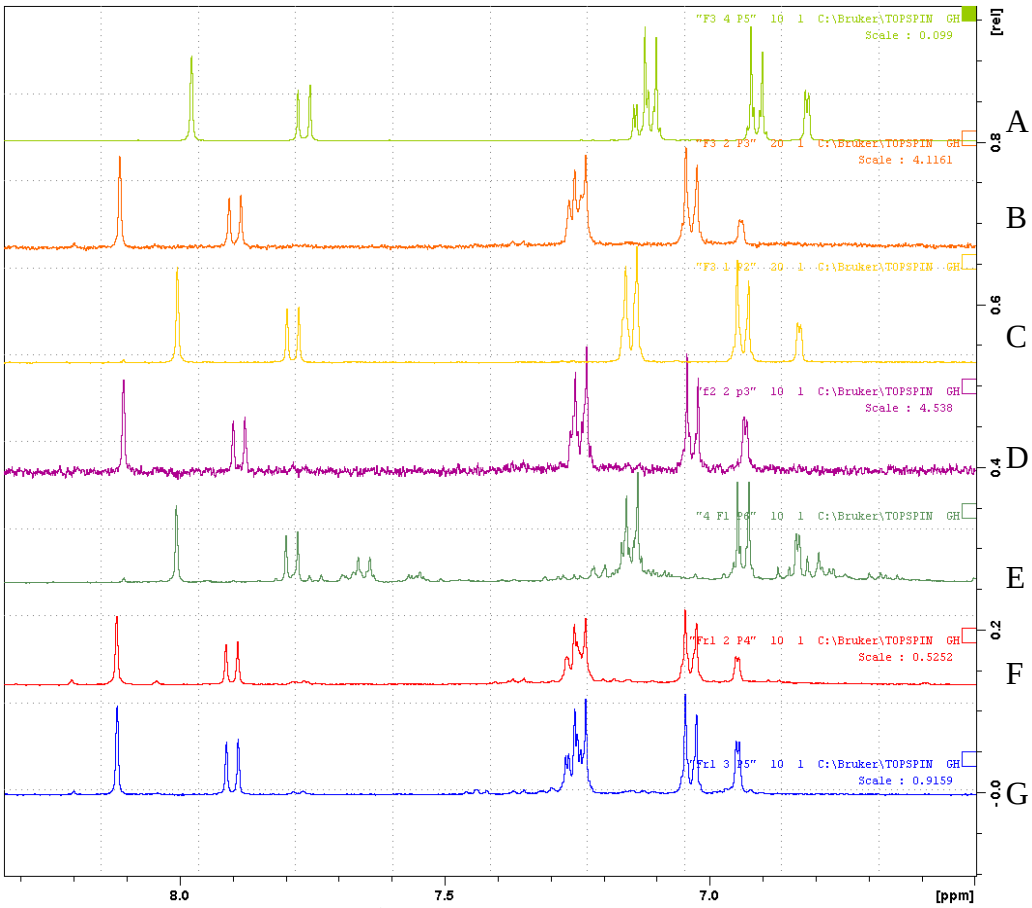


Figure 5.93: Comparison of ^{1}H NMR spectra for M1 F3 4 p5 (A) M1 F3 2 3 (B), M1 F3 1 p2 (C), M1 F2 2 p3 (D), M1 F1 4 p6 (E), M1 F1 2 p4 (F) and M1 F1 3 p5 (G).

M1 Fraction 3 5 p6

The NMR results of M1 F3 5 p6 are shown in Figures 5.94 to 5.96 and Appendix 3.122 to 3.29.

All the data were the same as for M1 F3 3 p4 (Data was not clear enough to define the confirmed structure).

The M1 F3 5 p6 was run on the better resolution NMR instrument at Cambridge University to get higher resolution data but the results were the same as the Salford University results (Figure 5.96 and Appendix 3.123 to 3.129).

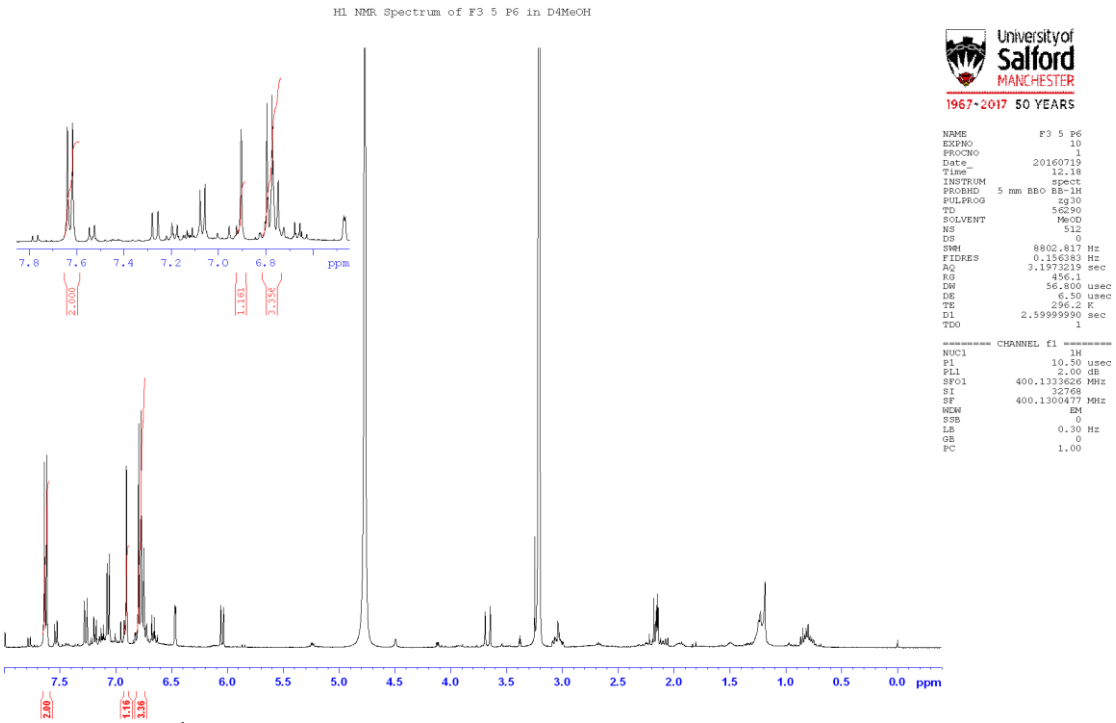


Figure 5.94: ^{1}H NMR Spectrum of M1 F3 5 p6 in CD_3OD+TMS

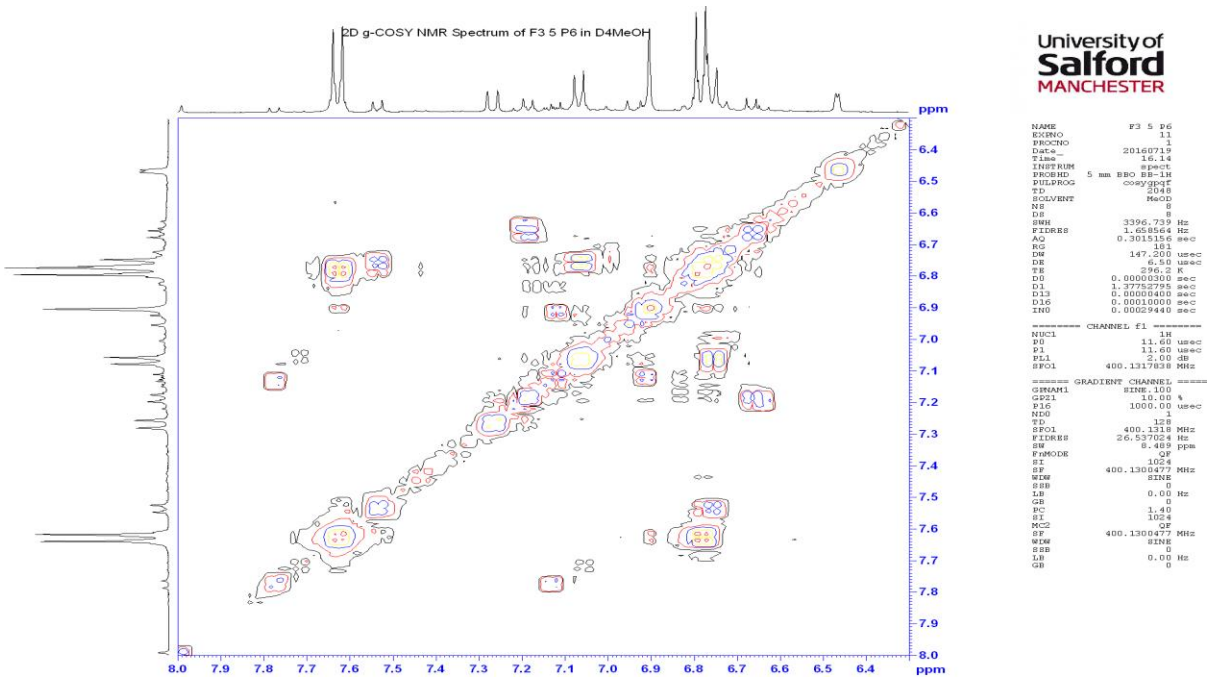


Figure 5.95: 2D g-COSY NMR Spectrum of M1 F3 5 p6 (expansion) in CD_3OD

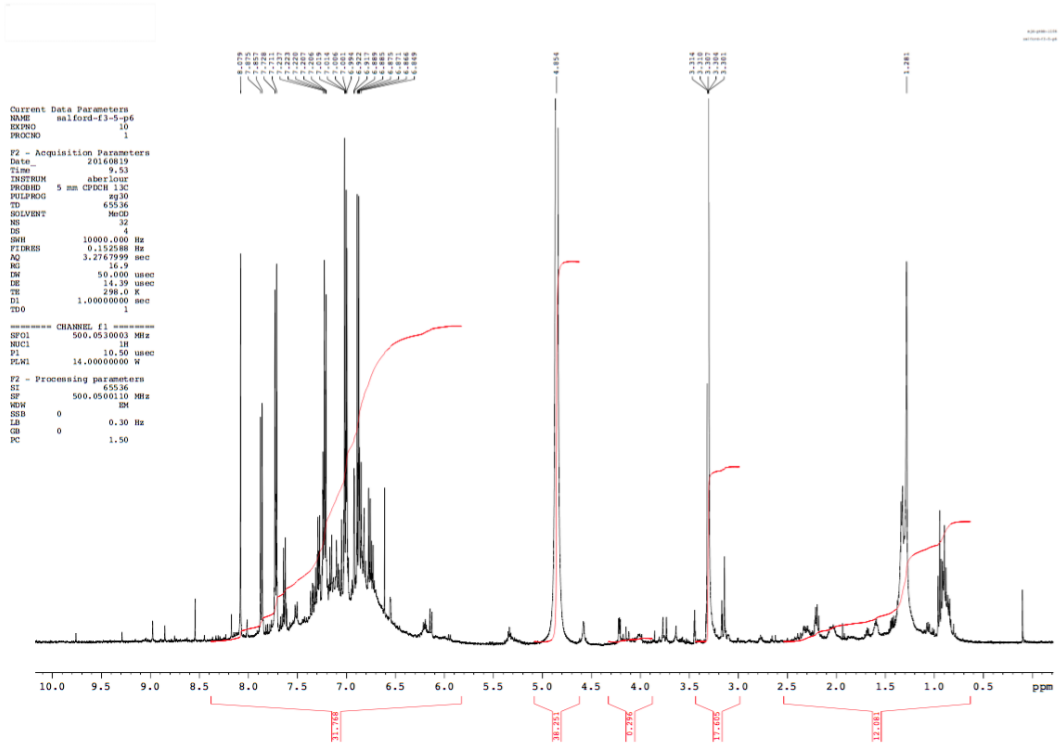


Figure 5.96: ^{1}H NMR Spectrum of M1 F3 5 p6 in CD_3OD+TMS

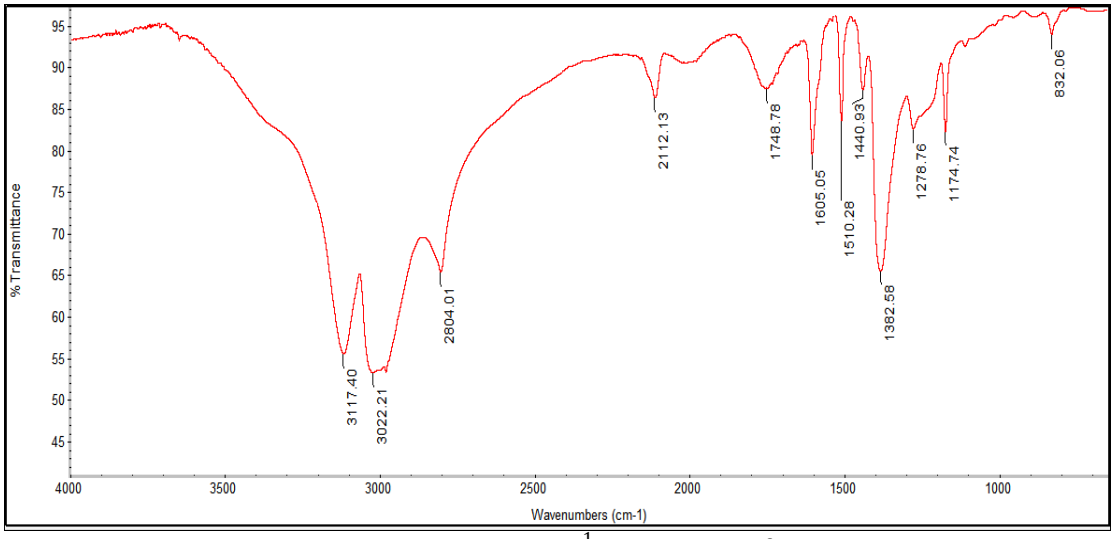


Figure 5.97: The FTIR spectra (ATR) 4 cm $^{-1}$ (16 Scans) of M1 F3 5 p6

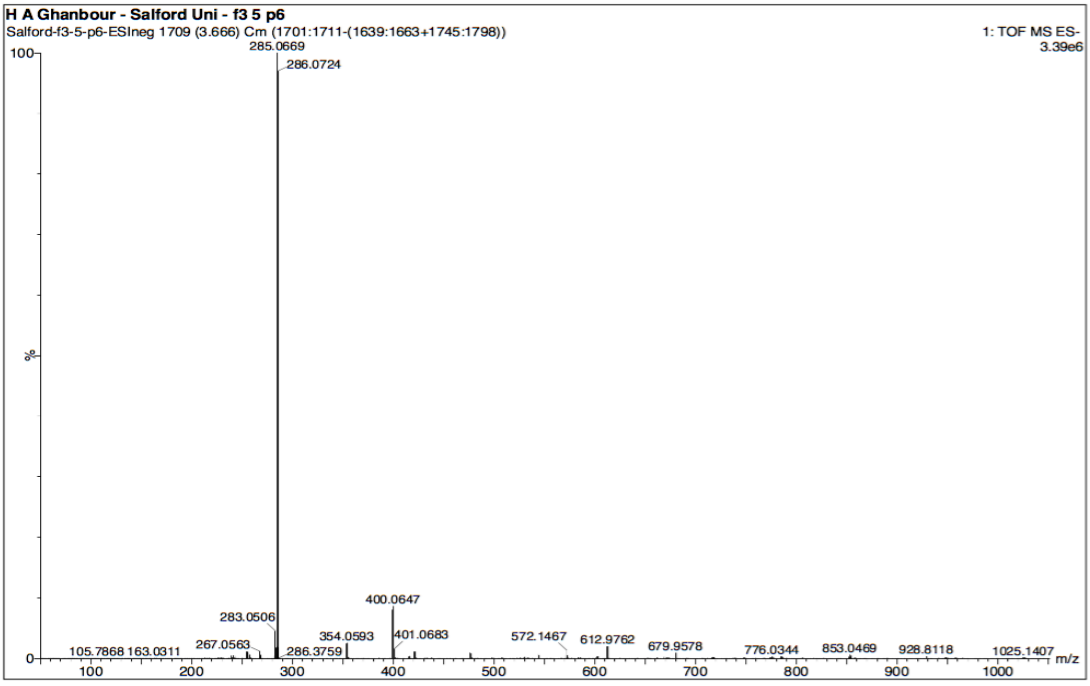


Figure 5.98: The negative ion spectrum from LC MS-TOF of M1 F3 5 p6

Summary of spectral data of compound M1 F3 5 p6

^{1}H-NMR (400 MHz, CD_3OD): δ H 7.63 (d of d), 6.91 (s), 6.79 (d of d)

^{13}C NMR (400 MHz, CD_3OD):

Due limited amount of material ^{13}C NMR was not obtained.

FTIR spectra (ATR) 4 cm $^{-1}$ (16 Scans): 3117(br), 3022(br), 2804(ms), 2112(ss), 1747(sbr), 1605(s), 1510(s), 1440(sms), 1382(ms), 1174(s)

MS: M/Z= 285 **(**M-H)$^{-}$

The IR, NMR and MS data are consistent with the structure shown in Figure 5.99 (2', 4', 5', 8 tetrahydroxyisoflavone).

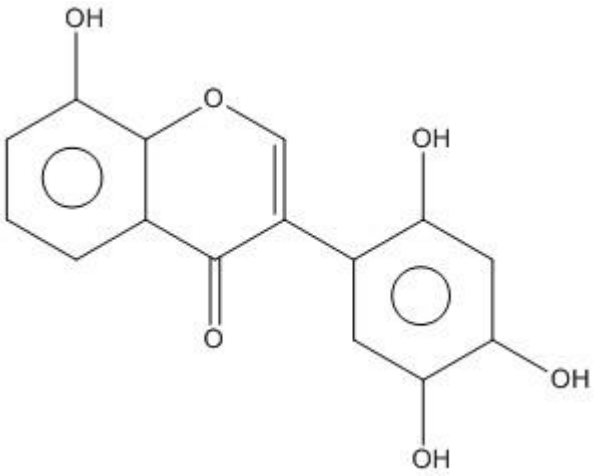


Figure 5.99: The proposed chemical structure of compound M1 F3 5 p6 ($C_{15}H_{10}O_6$, MW MZ=285 (M-H)-).

Stack plots of ^{1}H NMR spectra from M1 F3 3 p4 and M1 F3 5 p6 showed very similar spectra with different shifts on some peaks (Figure 5.100).

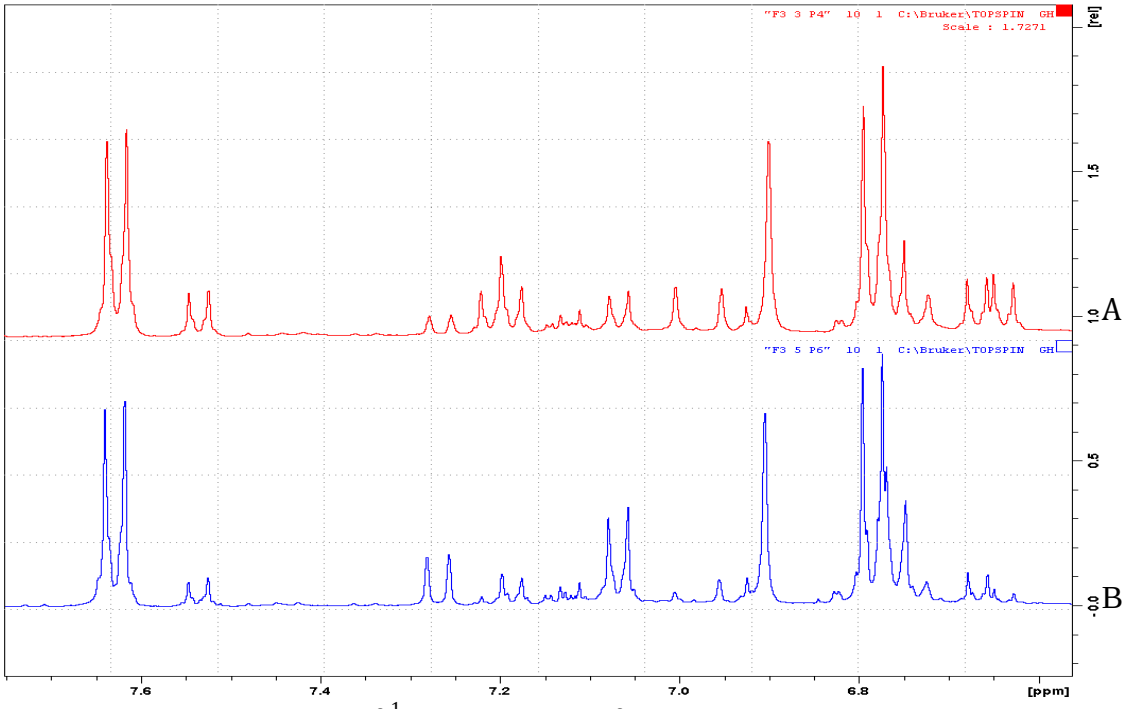


Figure 5.100: Comparison of ^{1}H NMR spectra for M1 F3 3 p4 (A) and M1 F3 5 p6 (B)

5.3 Discussion

5.3.1 Purification of the Antibiotics Produced from Isolates Es1 and M1 11

The results showed that it was possible to separate the components with antimicrobial activity despite the use of solid phase culture on agar. The extracts will have included cellular components and those present in the medium as well as the agents secreted by the cells which might have made their separations more difficult. However, in this study, RP-HPLC was used to fractionate the active components produced by cultures of two of the isolates Es1 and M111. HPLC was used in preference to TLC. TLC–bioautography combines TLC with both biological and chemical detection methods and has been widely used for the screening of organic extracts, mainly plant extracts, for antimicrobial activity e.g. see (Mehrabani *et al.*, 2013, Horváth *et al.*, 2010, Balouiri *et al.*, 2016). In the present study, TLC was tried for fractionation of the crude extracts but the results showed a high loss of antimicrobial activity (results not shown) and therefore HPLC was used. It was necessary to optimize the analytical HPLC separation conditions, namely choice of the best eluting solvent, column specifications and adjusting the solvent flow rate. To get semi-pure fractions of the biologically active crude extracts, reversed-phase HPLC was used with the separating solvent system (H_2O: MeOH, 30:70 v/v) which was found to give much better resolution and clearer peaks of the fractions. It has previously been recommended to not use mobile phases with less than 10 % organic solvent in water (Latif and Sarker, 2012).

HPLC is an extremely powerful and versatile technique. Any sample that can be dissolved in a liquid, HPLC has the ability to separate, identify, and quantitate the compounds that are present in this sample. RP-HPLC has been used extensively in the separation and identification of the bioactive compounds from extracts. One example was the fractionation of six bioactive compounds; terretrione D, methyl-2([2(1Hindol3yl)ethyl]carbamoyl)acetate, tryptamine, indole-3-carbaldehyde, 3,6-diisobutylpyrazin-2(1H)-one and terretrione C from *Penicillium* sp. CYE-87 (Shaala and Youssef, 2015), mahanine and mahanimbine alkaloids from *Murraya koenigii* (Pandit *et al*., 2011).

Analytical and preparative chromatography has been used in many studies of the separation and identification bioactive compounds from natural products. In early

stages of isolation and purification of the active compounds, analytical HPLC is commonly used for method development (Gupta *et al*., 2012). To produce higher yields of collected fractions, preparative and semi-preparative HPLC are used. Nowadays use of preparative HPLC has become an essential tool in isolation of most classes of natural products (Latif and Sarker, 2012).

In the RP-HPLC analysis the most common eluents used for natural products extracts are mixtures of aqueous and water-soluble organic solvent, methanol and acetonitrile are mostly used (Dolan, 1999). Use of a mobile phase of methanol-water mixture is the most commonly used RP- HPLC for the isolation of natural compounds. However, there is much published research that used mixture of water-acetonitrile in RP-HPLC natural products isolation. A survey of the period between 2008 and 2012 by (Bucar *et al*., 2013) showed that methanol-water mixture was employed as a solvent system in about half of natural products isolation. Sticher (2008) suggested that acetonitrile–water was better to use compared to other alcoholic solvents for RP-HPLC, due to its lower viscosity, thus giving reduced back pressure. However, Gilar *et al*. (2014), pointed out that acetonitrile was more costly, especially the highly pure solvent. Moreover, viscosity properties do not affect the separation quality and as it does not affect either the HPLC system or the column, they suggested that the methanol should be used instead of acetonitrile as a cheaper alternative with the same separation quality. Both Methanol–water and acetonitrile–water were used in the present study in a two stage purification process.

Once the HPLC fractionation had been optimized, fractions were collected and bio-assayed using the paper disc method and the *S. aureus* and *E.coli* to verify the antimicrobial activity of these fractions and the non-active fractions were not studied further. Such bioassay-guided isolation integrates the processes of separation of compounds in a mixture, using various analytical methods, with results obtained from biological testing. The first step of the process begins by testing a crude extract to confirm its biological activity presented in crude extract, followed by separation of the compounds in the extracts and *in-vitro* biological testing of purified compounds. In some fractions, further fractionation will be needed if the high purity level not obtained. Identification of chemical structures of the pure compounds is then performed (Jamil *et al*., 2012).

By using bioassay-guided isolation during the last few decades, many compounds have been isolated from different extracts and many of these have been reported to

have significant biological activities, some of which are of interest from the point of view of potential drug development (Khurram *et al*., 2015, Ejele *et al*., 2014, Kang *et al*., 2013).

Analytical column, HP Hewlett Packard Spherisorb ODS-2 5µm 250 mm x 4.6 I.D was used. One biologically active clear peak at retention time of 13.49 min gave the highest activity from the Es1 crude extract. It was observed that using these HPLC separation conditions, all fractions separated eluted before a retention time of approx. 15 min. Since the retention time of the largest peak area from M1 11 extracts was 13.58 min, this may indicate the possible similarity between the two components although obtained from two different fungal strains.

It was reported that, using HPLC, similar antimicrobial secondary metabolites are commonly produced from different spp. related to *Penicillium*. This was attributed mainly to some sort of genetic resemblance between them and between their specific metabolic pathways through which they can be produced (Martín *et al*., 2010)

Collectively, the results of HPLC analytical procedure for purification of the semi-purified extracts from Es1 revealed that the best separating solvent system was (H_2O: MeOH, 30:70 v/v), the best column was ACE Excel 5 C-18 amide column and three peaks numbered 3, 5 and 6 appeared at 12.23 (3), 27.27 (12) and 46.39 (46) min showed biological activity after being assayed using paper disc diffusion.

The mycelia and culture broth of the fungus *Penicillium* sp. CYE-87 were extracted with organic solvents in a study by Shaala and Youssef (2015). The combined crude extracts were subjected to partition by using RP- HPLC and the purification was performed by injecting crude extract into a C18 reversed-phase semi-preparative HPLC column.

For comparative purposes, the ethyl acetate extracts of the Es1 cultivated on AIA solid medium, AIB (broth) and mycelium (extracted by acetone) were subjected to two consecutive runs under same conditions, after which data of the three biological active fractions were recorded and compared. The results showed that, the three biological active fractions were present in all extracts with different concentrations. Using the area under the peaks as an indicator of concentration, fraction numbered 3, represented approx. 8.5%, 36% and 33.06% for solid, broth and mycelia as extraction sources. As regards fraction 5, its active components represented the highest percentage (17%) when being obtained by extraction with ethyl acetate from solid

AIA medium, while it was about 8.2% and 0.48% from AIB medium and its mycelia using acetone extracts, respectively. On the other hand, fraction 6 had the highest concentration as extracted from solid AIA medium (53.12%), while it reached about 16% and 22% from Es1 cultivated on AIB medium and extraction of mycelium with acetone, respectively. This is similar to a study by (Svahn *et al*., 2015) who reported that the three major extracts they prepared from *Penicillium nalgiovense Laxa*, the filtrate of the liquid culture, the mycelial growth in the liquid culture, and the cultures growing on solid medium, the result showed antimicrobial activity was present in all three extracts with different concentrations.

Preliminary results showed that active fractions from Es1 had four components and from M1 11 had ten components (see Table 5.5 and 5.7)

This part of work was a useful way to give more definite features about the number of biologically active components, degree of similarities and adjustment of the HPLC separation and purification conditions.

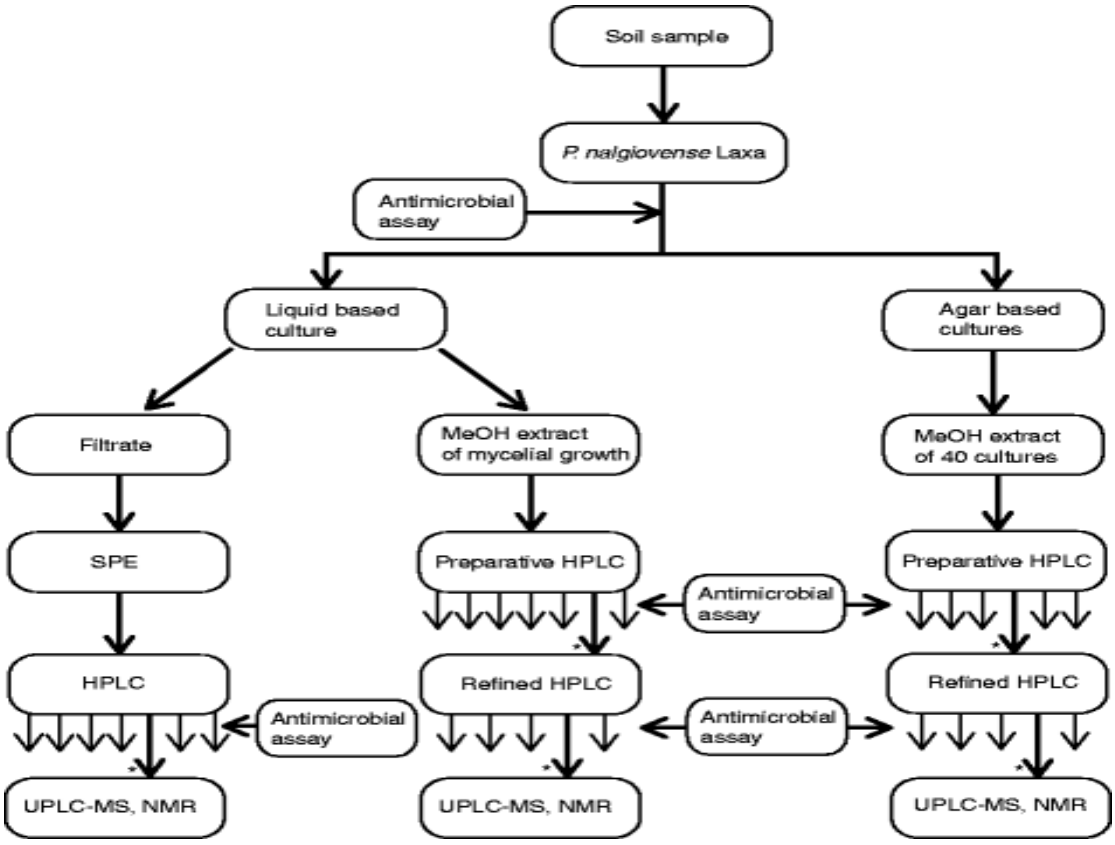


Figure 5.101: Model extraction procedure for cultivation, extraction, and isolation of metabolites with antimicrobial activity from *Penicillium nalgiovense* Laxa

Fractions without antimicrobial activity are marked with thinner arrows under the HPLC boxes, and those with antimicrobial activity are marked with an asterisk (*) modified from (Svahn *et al*., 2015).

A generalized scheme modified from (Svahn *et al.*, 2015) (Figure 5.101) was proposed to describe the stepwise procedure used to obtain pure fractions from organic extracts of biologically active components. These pure fractions were planned to be subjected to chemical structure elucidations using the approved modern techniques like proton and ^{13}C NMR and 2D g-COSY….etc. Some known compounds, have been isolated from *Penicillium* sp. CYE-87. The structures of the isolated compounds were established by spectral analysis, including 1D (^{1}H, ^{13}C) and 2D (COSY, multiplicity edited-HSQC and HMBC) NMR and HRESIMS, as well as

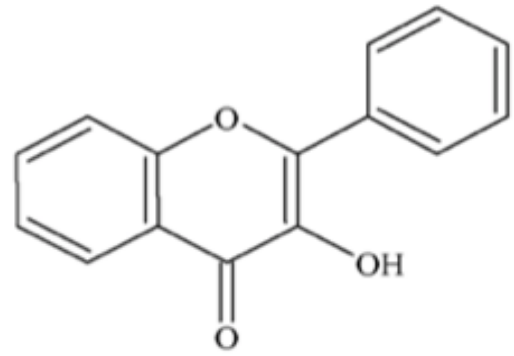
comparison of their NMR data with those in the literature. (Shaala and Youssef, 2015). In the present study similar stepwise procedures were followed.

5.3.2 Structure elucidation of the antibiotic(s) produced by isolates Es1 and M1 11

Spectrometry of the purified antibacterial compounds from the isolates Es1 and M1 11 was performed to characterize their chemical structures.

All fractions obtained were analyzed using ^{1}H NMR, 2D g-COSY, the infrared spectroscopy (IR) and MS for molecular weight determination. The elementary analysis for all samples was unfortunately not possible due to the lack of enough pure material of fractions. However, sufficient material was available for fractions Es1 5 Es1 6, M1 F3 3 p4, M1 F3 4 p5 and M1 F3 5 p6 these were therefore subjected to BBH1 decoupled ^{13}C analysis which indicated the total number of carbons included in the skeleton of compounds addition to JMOD ^{13}C, 2D g-HMQC and 2D g-HMBC analysis.

All the analytical data obtained using the above mentioned techniques indicated that the compounds Es1 F3/1 and Es1 F5 had similar structures with an extra oxygen in compound Es1 F3/1. Compounds Es1 F3/3 and Es1 F6 had very similar structure. On the other hand, compound M1 F3 3 p4 and M1 F3 5 p6 had very similar structures. The structures of the characterized compounds are summarized in Figures 5.102-5.103, for ease of comparison.



The skeleton structures of flavonol

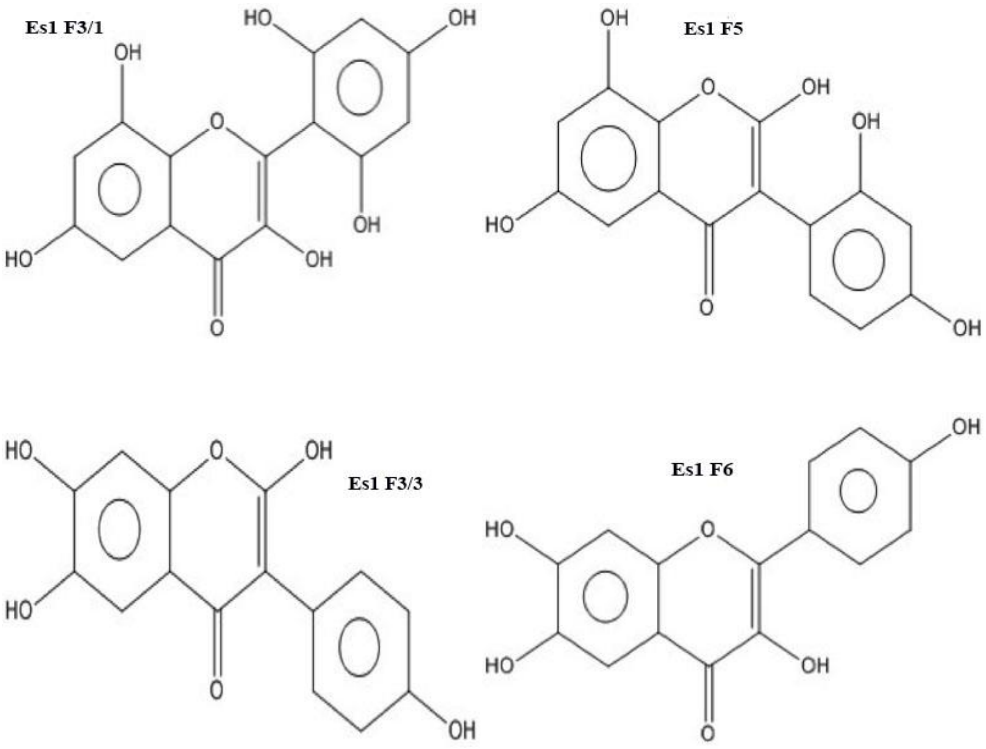
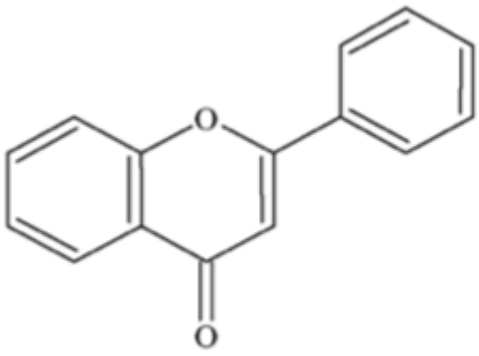


Figure 5.102: Summary of the chemical structures of fractions F3/1, F5, F3/3, F6, isolated from Es1 compared to skeleton structures of the flavonol



The skeleton structures of flavone

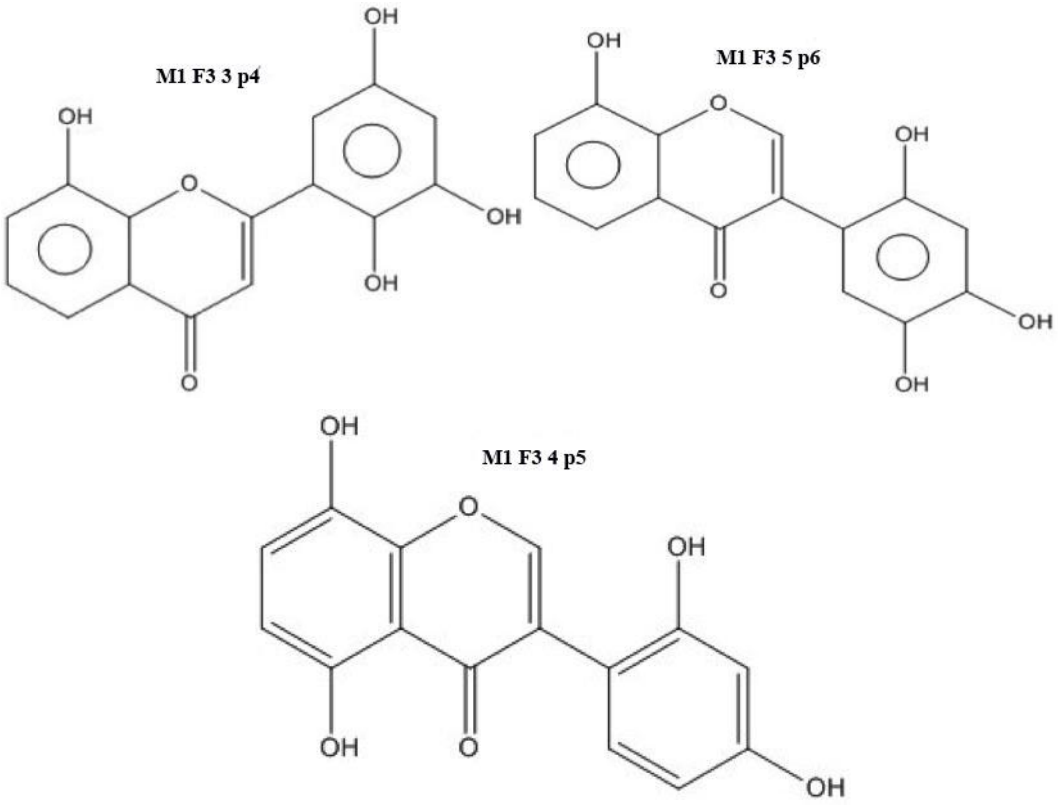


Figure 5.103: Summary of the chemical structures of fractions F3 3 p4, F3 5 p6 and F3 4 p5 isolated from M1 11 compared to skeleton structures of the flavone

Determination of the structures of seven of the fractions (Es1 fractions F3/1, F3/3, F5 and F6 and M1 11 fractions F3 3 p4, F3 4 p5 and F3 5 p6) showed that the active compounds were flavonoids and isoflavonoid. Three of the compounds were flavonoids and four were isoflavonoid. The fractions from Es1 were flavonols Es1 F3/1 (2′, 4′, 6′, 3, 6, 8- hexahydroxyflavone), Es1 F3/3 (1, 3′, 6, 7, tetrahydroxyisoflavone), Es1 F5 (2, 2', 4′, 6, 8, pentahydroxyisoflavone) and Es1 F6 (3, 4', 7, 8 tetrahydroxyflavone), whereas those from M1 11 were flavones M1 F3 3 p4 (1′, 3′, 4′, 8 tetrahydroxyflavone), M1 F3 4 p5 (2', 4', 5, 8 tetrahydroxyisoflavone) and M1 F3 5 p6 (2', 4', 5', 8 tetrahydroxyisoflavone). Unfortunately it was not possible to obtain sufficient amounts of the other fractions to allow full chemical structures to be determined. However, some of the properties of those fractions were similar to those that were fully characterized and may have represented a family of related compounds. Flavonoids are frequently isolated from plant sources (Cushnie and Lamb, 2011). Flavonoids are ubiquitous in photosynthesising cells and are commonly found in fruit, vegetables, nuts, seeds, stems, flowers, tea, wine, propolis and honey (Cushnie and Lamb, 2005) but, with the exception of endophytic fungi (Bhardwaj *et al.*, 2015a, Qiu *et al*., 2010) as far as the author is aware this is the first report of the production of such compounds from fungi from other sources.
Gossypetin (3,5,7,8,3,' 4'-hexahydroxyflavone) isolated from the flowers of Hibiscus sabdariffa, it has possesses excellent antimicrobial activity against *E. coli, S. aureus, B. subtilis, B. pumpilus and P. aeruginosa* (Mounnissamy *et al*., 2002) The structure of gossypetin similar to the flavonol that been isolated from Es1 (Es1 F3/1 (2′, 4′, 6′, 3, 6, 8- hexahydroxyflavone)) and the different is the position of the hydroxyl group.
Fisetin (3,3′,4′,7-tetrahydroxyflavone), can be found in various fruits and vegetables, such as strawberry, apple, persimmon, grape, onion, and cucumber, numerous researches have demonstrated the effects of fisetin against several diseases. It is reported to have neurotrophic, anticarcinogenic, anti-inflammatory, and other health beneficial effects (Khan *et al*., 2013), the structure of fisetin similar to the flavonol that been isolated from Es1 (Es1 F6 (3, 4', 7, 8 tetrahydroxyflavone)) and the different is the position of the hydroxyl group.
Luteolin (3',4',5,7 tetrahydroxyflavone) is a common flavone that found in many types of plants including fruits, vegetables. Having multiple biological effects such as anti-inflammation, anti-allergy and anticancer (Lin *et al*., 2008), the structure of luteolin

similar to the flavone that been isolated from M1 11 (M1 F3 3 p4 (1′, 3′, 4′, 8 tetrahydroxyflavone) and the different is the position of the hydroxyl group. Genistein (4',5,7- trihydroxyisoflavone) is an isoflavone which is the predominant isoflavone in legumes, such as soybeans, Numerous epidemiological studies have shown an inverse association between isoflavone consumption and the risk of breast cancer in Asians, indicating that dietary intervention may reduce the risk of breast cancer (Choi *et al*., 2013), (Peterson *et al*., 1991) report that genistein is a potent inhibitor of the growth of the human breast carcinoma cell lines (MDA-468 and MCF-7 and MCF-7-D-40) with IC_{50} values from 6.5 to 12.0 μg/ml, genistein has similar structures with isoflavones been isolated from M1 11 (M1 F3 4 p5 (2', 4', 5, 8 tetrahydroxyisoflavone) and M1 F3 5 p6 (2', 4', 5', 8 tetrahydroxyisoflavone)) that they have an extra oxygen and different position of hydroxyl group.
The recent combining of natural product chemistry and metabolomic approaches in drug discovery can certainly contribute to the development of new leads from marine derived fungi. (Nicoletti and Trincone, 2016). The results obtained in the present study show that terrestrial aquatic and land invertebrates are also potential sources of novel compounds.

The limitations of this part of the study were that many of the bioactive fractions were not characterized or structures determined due to the lack of the sufficient material and further studies would require larger amounts of purified fractions.

Chapter 6

Antimicrobial and anticancer activity

6.1 Introduction

6.1.1 Antimicrobial activity

Minimum Inhibitory Concentrations (MICs)

Minimum inhibitory concentrations (MICs) are considered the 'gold standard' for determining the susceptibility of organisms to antibiotic or antimicrobial agent and defined as the lowest concentration of a antimicrobial agent that prevents visible growth of a microorganism after 24 h incubation (for some organisms such as anaerobes this incubation time is extended as the organisms need more time to grow; Andrews, 2001). Microorganisms can be tested for their ability to produce visible growth on a series of agar plates (agar dilution), in tubes with broth (broth dilution), or in microplate wells of broth (broth microdilution) containing dilutions of an antimicrobial agent De Graaf, (2013). MICs are determined by dilution methods that used in relative testing of new antimicrobials agent, in diagnostic laboratories mainly to confirm unusual resistance, to confirm the susceptibility of organisms and to test those organisms when disc diffusion methods may be unreliable, for example when determining the susceptibility of coagulase- negative *staphylococci* to teicoplanin (Andrews, 2001).

6.1.2 Anticancer activity

Cancer is a broad term for diseases that are characterised by the uncontrolled proliferation of cells resulting from the disorder or dysfunction of regulatory signaling pathways that are naturally under tight control. Cancer can spread quickly and attack other tissues and organs and different cancers are recognized to have unique types or markers (McCauley *et al*., 2013). In countries all over the world cancer is a major public health hazard (Kinghorn *et al*., 2016). According to estimates from the International Agency for Research on Cancer (IARC), there were 14.1 million new cancer cases and 8.2 million cancer-related deaths in 2012 worldwide, which is predicted to increase to 23.9 million new cancer cases and 14.6 million cancer-related deaths by 2035 (Are *et al*., 2017).

Over 50 years natural products have played a major beneficial role in cancer chemotherapy. In Western countries over an approximately 70-year period of introduced anticancer drugs therapy out of a total of 175 small-molecule about 49% were obtained from natural products either obtained from organisms directly or other natural products (Kinghorn *et al*., 2016). For example, natural products of terrestrial microbial origin are used in cancer chemotherapy, include several anthracyclines (such as daunorubicin, doxorubicin, epirubicin, idarubicin, and valrubicin), bleomycin, actinomycin D and mitomycin C (Cragg *et al*., 2011). In turn, members of four classes of plant- derived anticancer agents are used clinically as antitumor agents, namely, the bisindole (vinca) alkaloids, the camptothecins, the epipodophyllotoxins, and the taxanes (Kinghorn *et al*., 2009.

An important aspect of the drug development for anticancer process is testing the natural product compounds for anticancer activity and cytotoxicity using human cancer cell cultures. To assess for preliminary anticancer activity, the MTT 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) *in vitro* assays are considered to be the most economic, reliable and convenient methods. This is based on their ease of use, accuracy and rapid indication of toxicity, as well as their sensitivity and specificity (McCauley *et al*., 2013).

The assay is based on the capability of mitochondrial dehydrogenase enzymes in living cells to change the yellow water-soluble substrate 3-(4,5- dimethylthiazol-2-y1)-2,5-diphenyl tetrazolium bromide (MTT) into a dark blue formazan product that is insoluble in water. Viable cells are able to metabolise the yellow MTT under tetrazolium ring cleavage to a water-insoluble purple-blue product which precipitates in the cellular cytosol and can be dissolved after cell lysis. However, cells that are dead following a toxic damage, cannot transform MTT. (Senthilraja *et al*., 2015)

The part of this study was to investigate the anticancer effects of compounds isolated from the Es1, M1 11 and La1a. In the present study, three human cell lines (two human breast cancer cell lines MDA-MB 468, MCF 7 and a control cell line of immortalized human keratinocytes, HACAT) were used for *in vitro* evaluation of anticancer activity.

The aims of this part of the study were to determine the MIC of the crude and purified antibiotic on bacteria and to determine their activity on cell culture.

6.2 Results

6.2.1 Antimicrobial activity

MIC's for *E.coli* and *S. aureus* (these organisms were selected as representatives of Gram negative and Gram positive bacteria as the amounts of antibiotic available were limited and further testing was not possible) were determined for crude and purified extracts by the broth micro-dilution method (Andrews, 2001) and the results are shown in Tables 6.1-6.3. The crude extracts had higher activity against *S. aureus* than *E. coli*. MIC values on *E. coli* were 2 (M1 11) and 4 (La1a) times higher than the effective dose of Es1 crude extract which had the highest antimicrobial activity with an MIC value of 0.39 µg/ml against *S. aureus* (Table 6.1).

The MIC's of fractions of Es1 (F3/1, F3/3, F5 and F6) were determined (Table 6.2). Fraction F3/1 had the highest value on both test organisms (6.25 µg/ml each). Fraction 6 had the best antimicrobial activity with MIC of 0.78 µg/ml on *E. coli* and 0.39 µg/ml on *S. aureus*) similar to the crude extracts. Fractions F5, F3/3 and F3/1 had lower activities which were 2, 4 and 8 x the MIC of F6 (Table 6.2).

The only fractions of M1 11 with activity similar to the crude preparation was F3 4 p5 (3.13 µg/ml *E. coli* and 1.65 µg/ml on *S. aureus*: Table 6.3). All other fractions (F1 1 p3, F1 2 p4, F1 3 p5, F1 4 p6, F3 1 p2, F3 2 p3 and F3 3 p4) had lower antibacterial activities with MIC values between 6.25 and 50 µg/ml.

Table 6.1: The Minimal Inhibitory Concentrations (µg mL^{-1}) of the crude extracts of the isolates ES1, M1 11and La1.

Extract	*E. coli*	*S. aureus*
Es1	0.78	0.39
M1 11	1.56	0.78
La 1 a	3.13	1.56

Determined by the microdilution plate method using AIB medium (Andrews, 2001)

Table 6.2: The Minimal Inhibitory Concentrations (µg mL^{-1}) of the Es1 fractions

Es1 Fractions	Test organisms	
	E. coli	*S. aureus*
F3/1	6.25	6.25
F3/3	3.13	1.56
F5	1.56	0.78
F6	0.78	0.39

Determined by the microdilution plate method using AIB medium (Andrews, 2001)

Table 6.3: The Minimal Inhibitory Concentrations (μg mL^{-1}) of the M1 11 fractions

M1 11 Fractions	*E. coli*	*S. aureus*
F1/1 p3	50	25
F1/2 p4	12.5	6.25
F1/3 p5	12.5	12.5
F1/4 p6	25	12.5
F3/1 p2	25	12.5
F3/2 p3	25	12.5
F3/3 p4	12.5	6.25
F3/4 p5	3.13	1.56
F3/5 p6	12.5	6.25

Determined by the microdilution plate method using AIB medium (Andrews, 2001)

6.2.2 Anticancer activity

MDA-MB 468, MCF7 and HACAT cell lines were used to test the compounds for anticancer activity and cytotoxicity (again amounts of antibiotic were limited and three strains of cell line, two cancer cell lines and one normal cell line were therefore used). MTT viability assays were conducted as described in section 2.18.2, then Ascent software - Thermo Labsystem Multiskan Ascent plate reader was used to measure absorbance at 540 nm and calculate the inhibitory concentration (IC_{50}). Figures 6.1- 6.5 show the growth inhibition curves and the IC_{50} values are summarised in Tables 6.4 and 6.5. The results showed that the crude extracts of Es1, M1 11 and La1a were found to have MDA-MB 468 IC_{50} values ranging from 13-21 μg/ml. IC_{50} values were >100 μg/ml with the MCF7 and HACAT cell lines (data not shown). In contrast, the purified fractions F6 and F3/3 were found to have HACAT IC_{50} values of 9 and 6 μg/ml respectively, lower than the crude extracts (Table 6.5).

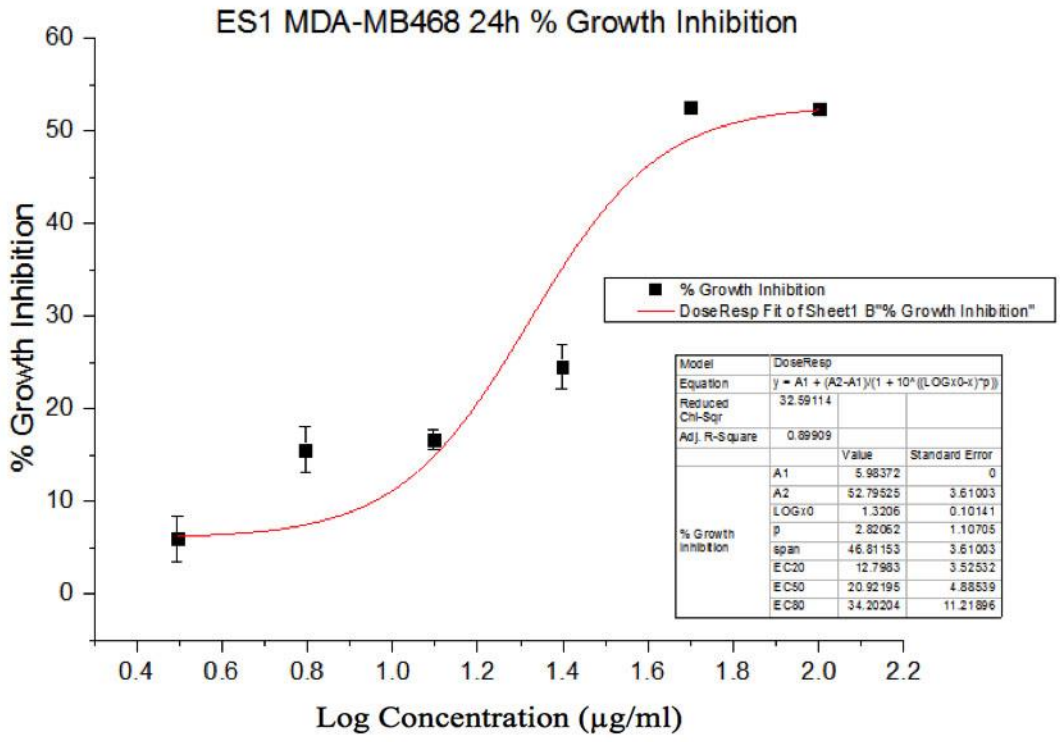


Figure 6.1: Representative IC_{50} curve showing % growth inhibition effect of Es1 extracts treatment on the MDA-MB 468 cell line.

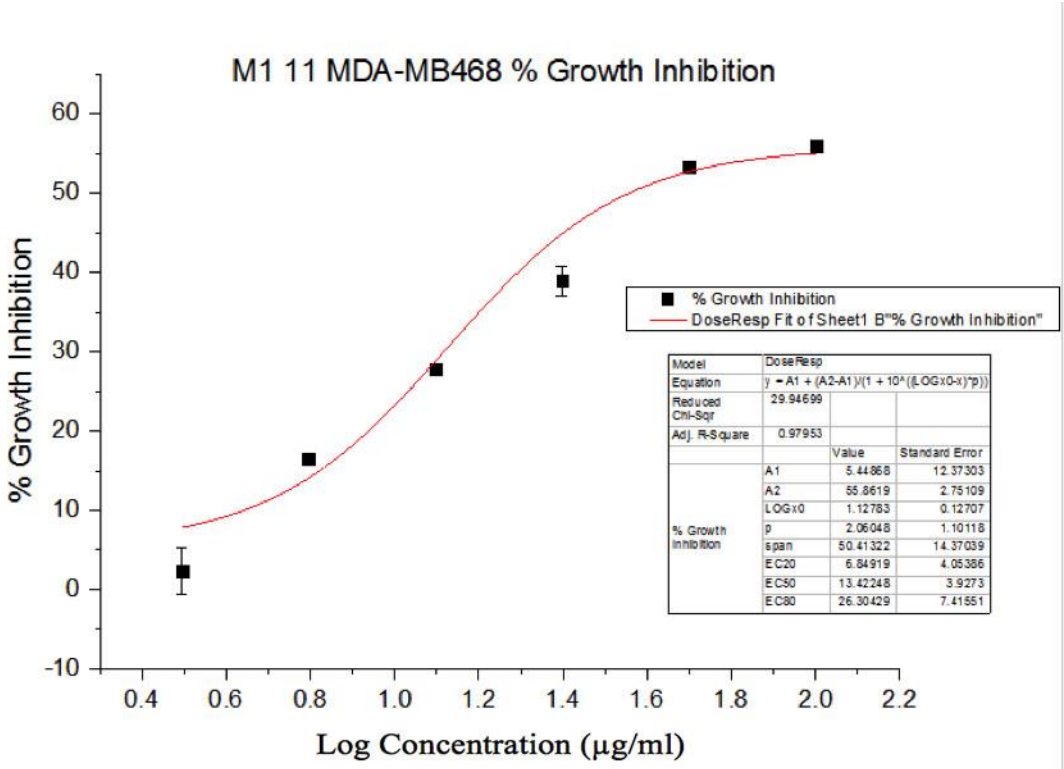


Figure 6.2: Representative IC_{50} curve showing % growth inhibition effect of M1 11 extracts treatment on the MDA-MB 468 cell line.

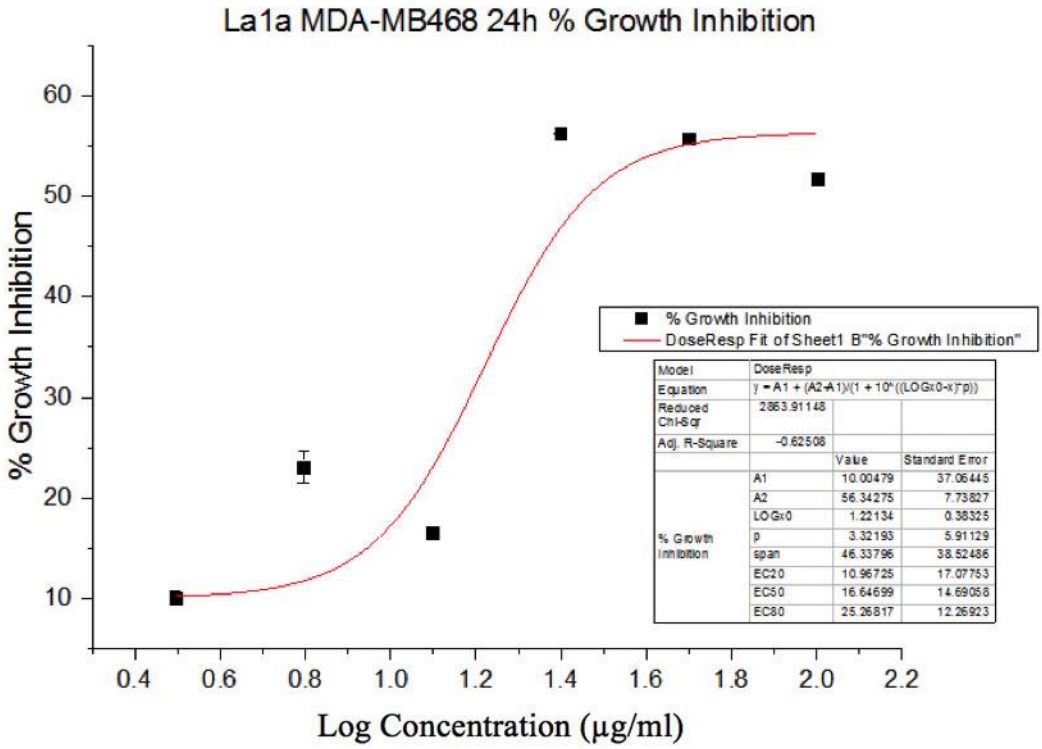


Figure 6.3: Representative IC_{50} curve showing % growth inhibition effect of La1a extracts treatment on the MDA-MB 468 cell line.

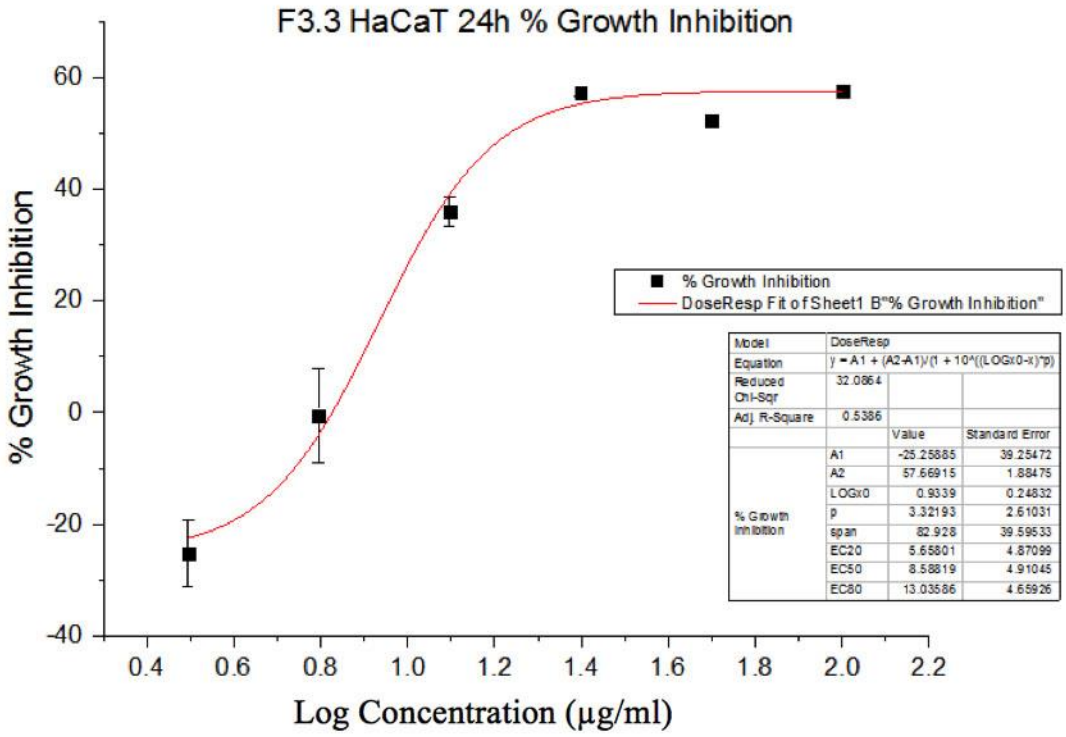


Figure 6.4: Representative IC_{50} curve showing % growth inhibition effect of F3/3 compound treatment on the HACAT cell line.

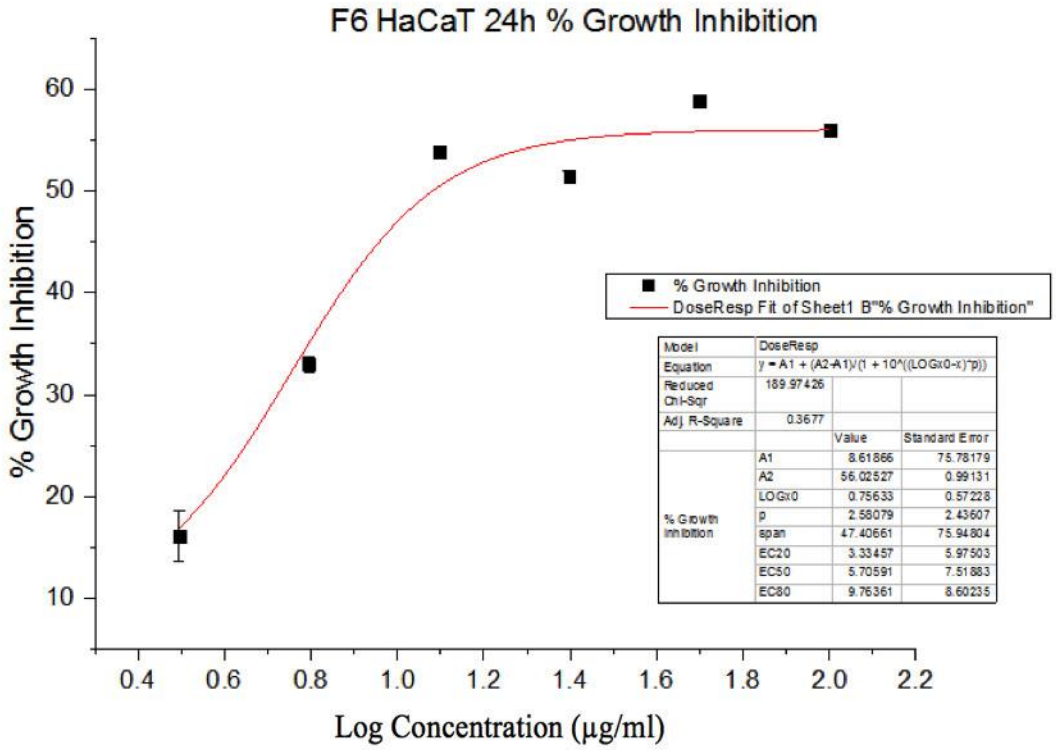


Figure 6.5: Representative IC_{50} curve showing % growth inhibition effect of F3/3 compound treatment on the HACAT cell line.

Table 6.4: MDA-MB 468 IC_{50} values and standard error when treated with extracts of Es1, M1 11 and La1a

MDA-MB 468		
Extracts	IC_{50} (µg/ml)	Standard Error
Es1	21	5
M1 11	13	4
La1a1	17	15

Table: 6.5: HACAT IC_{50} values and standard error values when treated with fractions F6 and F3/3

HACAT		
Compound	IC_{50} (µg/ml)	Standard Error
F3/3	9	5
F6	6	8

6.3 Discussion

6.3.1 Antimicrobial activity

The results showed that crude extracts from all three strains showed higher activity against *S. aureus* than *E. coli*. MIC values for M1-11 and La1a crude extracts on *E. coli* were 2 to 4 times higher than that for Es1 extract. The Es1 crude extract was had the lowest MIC values on both *E. coli* and *S. aureus* at 0.78 and 0.39 µg/ml. This is similar to the reported MIC for Chrysoeriol (flavone) extracted from *Capsicum frutescens* (whole fruits, peel and seeds) against *E. coli* and *S. aureus* at 0.06 and 0.25 µg/ml respectively (Nascimento *et al*., 2014), and is also similar to antibiotics used clinically against *S. aureus* e.g. gentamicin (0.125-0.25 µg/ml; (Sørensen and Sørensen, 1993); 0.5-1 µg/ml (Tam *et al*., 2006))

All four fractions of Es1 were active with F3/1< F3/3<F5 <F6 and F 6 had antimicrobial activity similar to the crude preparation. These results are in line with results for the published MIC for flavonoids. (Table 6.6)

Table 6.6: MIC of some natural flavonoids

Flavonoid	MIC (µg/mL)	
	Gram-positive	Gram-negative
Panduratin A	0.06–2.0	N/T
Isobavachalcone	0.3–0.6	0.3 to >39.1
Bartericin A	0.6–2.4	0.3–39.1
Scandenone	0.5–8	2–32
Kaempferol 3-*O*-α-l-(2″,4″-di-*E*-*p*-coumaroyl)-rhamnoside	0.5 to >16	>16
Sepicanin A	1.2	N/T
Isolupalbigenin	1.6–3.1	N/T
Flavone	7.8–31.3	1.95–31.3
3′-*O*-methyldiplacol	2–4	>32
Licochalcone A	2–8	N/T
Chrysoeriol	0.25	0.06

Modified from (Cushnie and Lamb, 2011, Nascimento *et al*., 2014)

Fractions of M1 11 were comparatively weaker with MIC values between 6.25 and 50 µg/ml except for F3 4 p5 with MIC of 3.13 µg/ml *E. coli* and 1.65 µg/ml on *S. aureus* The results showed that the resulting fractions were less active individually than the crude extract of M1 11 possibly indicating some synergistic effects between the fractions.

6.3.2 Anticancer activity

Flavonoids are a class of plant secondary metabolites possessing a broad spectrum of pharmacological activity including anticancer activities. Flavonoids have diversity of structural patterns this diversity has resulted in flavonoids being recognized as a rich source of compounds with potential anticancer properties (Ravishankar *et al*., 2013) In this study whole extracts of Es1, M1 11 and La1a were found to have anticancer activity against MDA-MB 468 cell line and showed IC_{50} values in the low microgram per millimeter (from 13 to 21 µg/ml) but IC_{50} values were >100 µg/ml with the MCF7 and HACAT cell lines. This indicates that they might be useful anti-infective agents as they may show low cytotoxicity and the potential for use as an anticancer therapy is clearly worth investigating further. (Peterson *et al*., 1991) examined the effect of isoflavones (genistein, biochanin A and daidzein) on the growth of the human breast carcinoma cell lines, MDA-468, and MCF-7 and MCF-7-D-40 and reported that Genistein was a potent inhibitor of the growth of the human breast carcinoma cell lines (MDA-468 and MCF-7 and MCF-7-D-40) with IC_{50} values from 6.5 to 12.0 µg/ml, whereas biochanin A and daidzein are weaker growth inhibitors (IC_{50}values from 20 to 34 µg/ml).

The purified fractions except Es1 F3/3 and Es1 F6 were found to have IC_{50} values>100 µg/ml, High IC_{50} and low MIC may indicate fractions suitable for testing for treatment of human infections. The fractions Es1 F6 and Es1 F3/3 were found to have HACAT IC_{50} values of 9 and 6 µg/ml respectively and may be too cytotoxic for use as treatment for bacterial infections. Further testing was not possible due to limited amounts of materials and a much larger scale preparation would be necessary to provide enough material.

The limitation of this part of the study were that only a few organisms and cell lines were tested due to the limited amount of the antibiotics (just two bacteria and three cell lines were tested), if much larger amount antibiotics were available more test organisms and cell lines could be tested.

Chapter 7

General discussion and conclusions

The objectives of this study were to investigate terrestrial and aquatic invertebrates for the presence of antibiotic producing microorganisms and to isolate the organisms, screen them for activity against Gram-positive and Gram-negative bacteria. The results showed that this was a promising source for antibiotic producing organisms and it was possible to isolate antibiotic producing strains from invertebrates from both sources. Whether the microorganisms that were present in the invertebrates had originated in the environment e.g. washed in from surrounding soils and ingested or were present on food ingested by the aquatic and terrestrial invertebrates or whether they were associated with the invertebrates is not clear. Much of the previous work on terrestrial invertebrates has been on social insects e.g. ants (Mueller *et al*., 2008), termites (Rohland and Meyers, 2015) wasps (Kaltenpoth *et al.,* 2005) and millipedes, all of which have been shown to harbour antibiotic producers (Liu *et al*., 2016). The present study included different kinds of invertebrates. Forty-seven antibiotic producers were isolated from terrestrial and aquatic invertebrates. On the primary screening program the results indicated that three strains isolated from *Helix aspersa* (Es1), *Lumbriculus variegata* (La1a) and *Baetis* larvae (M1 11) were selected for further study as they had broad spectrum antibacterial activity. When the isolates were subjected to PCR and sequencing of 16S and 18S rDNA for identification, this showed that the majority were in fact fungi; mainly *Penicillium* spp. and only 5 were *Streptomyces* spp. Although the media used were designed to isolate streptomycetes, just 11% of the isolated antibiotic producing microorganisms were *Streptomyces* spp. No antifungal agents were added to the media and, in fact, 89% of the isolated antibiotic producing microorganisms were fungi (the morphology of the fungi and streptomycetes colonies on the AIA were similar and were identified by molecular methods). On the secondary screening program the three isolates Es1, M1 11 and La1a which had best activity on the primary screening showed strong activity against Gram-positive and Gram-negative bacteria including MRSA and were therefore chosen for further study. Unfortunately it was not possible in the time available to test

all isolates especially isolates with narrow spectrum activity against Gram-negative bacteria only which may be useful therapeutically.

For further identification of the organisms to species level, the isolates (Es1, M1 11 and La1a) were sent to NICMB Limited Company, to sequence ITS region to distinguish between species because it has recently been accepted as the official sequence for identification of fungi (Schoch *et al*., 2012). The results confirmed that the isolate M1 11 was *P. chrysogenum; Es1 and La1a* were closely related and were probably also strains of *P. chrysogenum*.

Isolate *P. chrysogenum* Es1 was chosen for secondary screening which included comparative cultivation in different types of growth media seeking optimal antibiotic production. Although the media used were designed for bacteria, the antibiotic activity was high and therefore media used for fungi were not used. Unfortunately, there were no lab facilities to scale up this process using liquid media and therefore, the work was re-planned to grow it on solid AI agar medium. This was rather time- and effort-consuming, however, antimicrobial material has previously been successfully isolated from solid medium (Svahn *et al*., 2015, Pandey *et al*., 2000). The process was suitable for scale-up. Following fractionation of the crude extracts, four pure fractions were obtained from Es1 crude extract (F3/1, F3/3, 5 and 6) and ten pure fractions from M1 11 (F1 1 p3, F1 2 p4 F1 3 p5, F1 4 p6, F2 2 p2, F3 1 p2, F3 2 p3, F3 3 p4, F3 4 p5 and F3 5 p6), all pure fractions analyzed using ^{1}H NMR, 2D g-COSY, the infrared spectroscopy (IR) and MS for molecular weight determination.

The elementary analysis for all samples was unfortunately not possible due to the lack of enough pure material of nine fractions. Meanwhile, the relative abundance of F5 (10 mg) and F6 (24 mg) from Es1 isolate and F3 3 p4 (3 mg), F3 4 p5 (8 mg) and F3 5 p6 (4 mg) from M1 11 isolate, enabled further chemical analyses, BBH1 decoupled ^{13}C analysis which indicated the total number of carbons included in the skeleton of compounds addition to 2D g-HMQC and 2D g-HMBC techniques. Finally, these two fractions were analyzed by JMOD C13 analysis.

The structure elucidation of the antibiotics obtained from the isolated organisms are conclusive and revealed that the compounds obtained from Es1 and M1 11 were flavonoids, three flavones and four isoflavones. As far as the author is aware the structures are unique and have not previously been isolated. These types of compounds are frequently isolated from plant sources (Cushnie and Lamb, 2011,

Cushnie and Lamb, 2005) but, with the exception of endophytic fungi (Bhardwaj *et al*., 2015a, Qiu *et al*., 2010), as far as the author is aware this is one of the first reports of the production of such compounds from fungi from other sources. It is possible that they represent endophytic fungi obtained from the food eaten by the invertebrates which includes plant material. *Lumbriculus* feed on decaying leaves and wood and ingest microorganisms, *Helix* sp. feed on plant materials and *Baetis* larvae feed on detritus and algae.

Unfortunately it was not possible to obtain structures for all pure fractions due to the lack of enough material. However, some of the properties of those fractions were similar to those that were fully characterized and may have represented a family of related compounds.

Examination of the genome for PKS genes would confirm the biosynthetic capabilities of the fungi. Juvvadi *et al*., (2005) discovered chalcone synthase (CHS) genes belonging to the type III PKS in *Aspergillus oryzae*. CHS genes are known to play a vital role in the biosynthesis of flavonoids in plants. CHSs in plants known to catalyze the first committed step in the phenylpropanoid pathway leading to the biosynthesis of flavonoids, isoflavonoids and anthocyanins by the condensation of 4-hydroxycinnamoyl-CoA and three malonyl-CoA molecules to the chalcone derivative, naringenin chalcone (Figure 7.1: Ferrer *et al*., 1999).

Until recent years it was believed that type III PKSs (PKSs are classified into three groups: types I, II, and III) existed exclusively in plants and bacteria, but recently several fungal genome sequencing studies revealed the presence of type III PKSs in filamentous fungi such as *Aspergillus oryzae*, *Saccharomyces cerevisiae, Schizosaccharomyces pombe,* and *Neurospora crassa* (Yu *et al*., 2012).

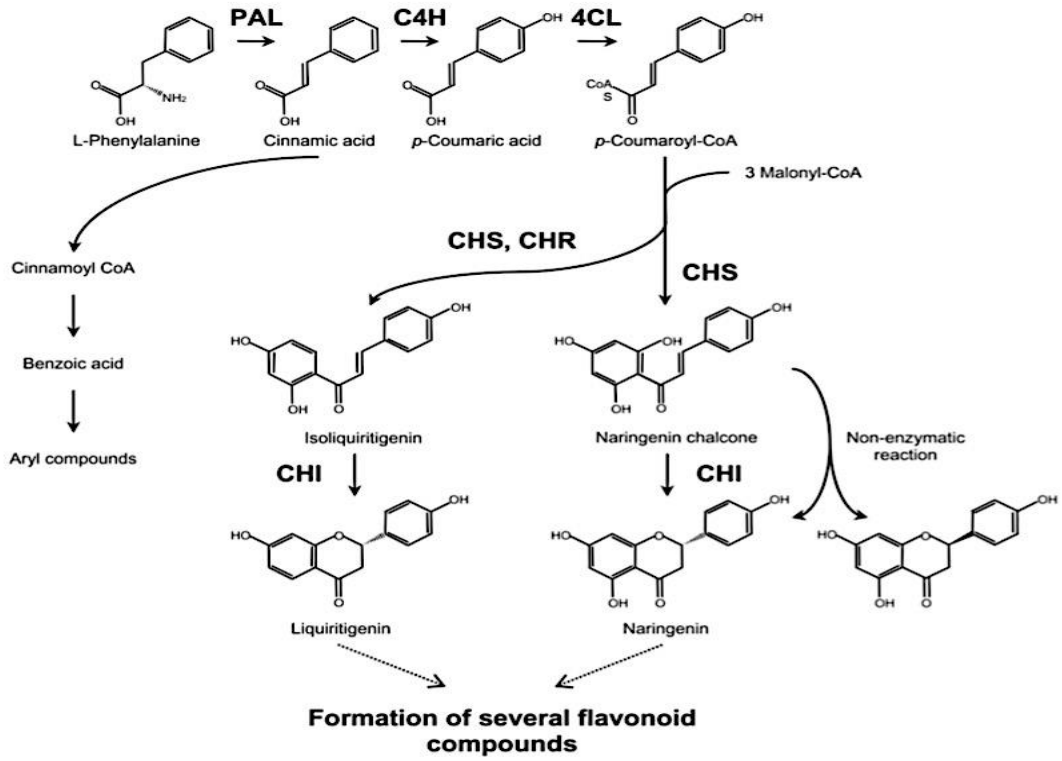


Figure 7.1: Flavonoids biosynthesis pathway modified from Juvvadi *et al*. (2005)

PAL: Phenylalanine ammonia-lyase; C4H: cinnamate-4-hydroxylase; 4CL: 4-coumaroyl: CoA-ligase; CHS: chalcone synthase; CHI: chalcone isomerase; CHR: chalcone reductase.

Preliminary examination for anticancer activity showed that crude extracts of Es1, M1 11 and La1a may have some activity. This indicates that the potential for use as an anticancer therapy is clearly worth investigating further. The purified fractions except F3/3 and F6 were found to have IC_{50} values>100 µg/ml, High IC_{50} and low MIC may indicate fractions suitable for testing for treatment of human infections because they may have low toxicity.

Overall, this investigation suggests that terrestrial and aquatic invertebrates are a rich source of antibiotic producing microorganisms. The antimicrobial activity of the pure compounds showed that they had high activity against Gram positive and Gram negative bacteria with low MIC values, which may represent a potential for pharmaceutical and/or agricultural applications. It is hoped that these components when isolated into pure constituents can serve as leads for the development of novel and potent antibiotics.

Further work

The availability of some instrumentation would have improved the work of this study, for example if preparative HPLC were available for purification of the bioactive compounds, this would save much time for collecting the bioactive fractions. Also fermentation facilities to cultivate cultures of relatively large volumes of 10 L+ / run, would facilitate production of the crude extract and make the fractionation easier as there would probably be fewer contaminants when using cell free filtered medium than extraction from solid culture.

The results of the antibacterial evaluation of the 47 isolates suggested that future work should include investigating the active compounds from the rest of the organisms in this study by using different extracting solvents with different polarities to retrieve as many active compounds as possible. This study has proved that the pure compounds obtained from Es1 and M1 11 have high antibacterial activity but the mechanism of action was not investigated due the limitation of the study time. Further studies would include determining the mode of action (MOA) of the compounds.

The macromolecular synthesis assays (MMS) one of the most popular methods in MOA determination. MMS assays uses radioactively labeled precursors to determine whether a compound specifically inhibits protein, RNA, DNA, lipid, or peptidoglycan synthesis (Nonejuie, 2014, Nonejuie *et al*., 2013) by introduction of a radiolabeled precursor of a particular macromolecule into bacterial culture treated with antibacterial compound. This precursor is allowed to incorporate into its corresponding macromolecule for ten minutes of incubation, after which macromolecules are precipitated by the addition of trichloracetic acid and isolated by filtration, liquid scintillant add to filters, radiolabeled incorporation measure using a Chameleon multi label plate scintillation counter (Gerits *et al*., 2016), inhibition of the macromolecular process by the antibacterial compound is indicated by diminished levels of radioactivity (Low radioactive count). For example, it is possible to monitor inhibition of DNA, RNA, or protein synthesis by the addition of either [methyl-^{3}H]thymidine, [5,6-^{3}H] uridine or L-[G-^{3}H]glutamine respectively to the bacterial cultures and determining whether there was any inhibition of incorporation into macromolecules as described by Gerits *et al*. (2016).

Effects on membrane permeability can be determined by using SYTOX green stain as described by (Yaraksa *et al*., 2014, Gerits *et al*., 2016), this stain only enters the

cytoplasm if the membrane is compromised. Upon uptake, SYTOX green binds to nucleic acids, which in turn results in increased fluorescence (Roth *et al*., 1997). Previous studies on other flavonoids has shown the mode of action of selected flavonoids. The activity of quercetin, for example, has been at least partially attributed to inhibition of DNA gyrase, sophoraflavone G and (-)-epigallocatechin gallate inhibit cytoplasmic membrane function, and licochalcones A and C inhibit energy metabolism (Cushnie and Lamb, 2005).

Further investigations are also needed for confirmation of the identification of *Penicillium* species by sequencing other genes e.g. β-tubulin (BenA) gene because there are clearly problems identifying *Penicillium* species by sequencing ITS alone. Further studies will also be needed to confirm whether the microorganisms were present in the invertebrates having been obtained from the environment or whether they were these as part of a symbiotic or other association. As far as the author is aware there is no evidence for symbiotic associations of invertebrates with *Penicillium* sp. but they are present as endosymbionts in plants. Further work could involve testing more examples of the invertebrates to see whether the microorganisms were always isolated which might indicate a symbiotic association.

Chapter 8

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Chapter 9

Appendices

Section 1: sequence alignments

Appendix 1.1: Sequence alignments for La2 isolate with the three closest identification strains

```
Query  38    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  97
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1617  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1676

Query  98    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  157
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1677  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1736

Query  158   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  206
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1737  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  1785
```

Uncultured *Penicillium* CHiv76 18S

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Query  38    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  97
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3     GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  62

Query  98    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  157
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  63    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  122

Query  158   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  206
             |||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  123   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA  171
```

Uncultured fungus clone Aerocomp_EO40

```
Query  38    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  97
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1628  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1687

Query  98    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  157
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1688  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1747

Query  158   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  206
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1748  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  1796
```

Penicillium commune strain IBT 15141

Appendix 1.2: Sequence alignments for La4 isolate with the three closest identification strains

```
Query  29    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  88
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1628  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1687

Query  89    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  148
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1688  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1747

Query  149   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  197
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1748  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  1796
```

Penicillium tardum strain KCTC16051

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Query  29    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  88
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1617  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1676

Query  89    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  148
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1677  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1736

Query  149   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  197
             |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1737  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  1785
```

Penicillium chrysogenum strain 215

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Query  29    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  88
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1627  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1686

Query  89    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  148
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1687  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1746

Query  149   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  197
             |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1747  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  1795
```

Penicillium expansum

Appendix 1.3: Sequence alignments for La5 isolate with the three closest identification strains

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Query  30    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  89
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  90    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  149
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Sbjct  1687  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1746

Query  150   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  198
             |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1747  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  1795
```

Eupenicillium crustaceum

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Query  30    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  89
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1608  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1667

Query  90    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  149
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1668  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1727

Query  150   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATC  197
             |||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1728  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATC  1775
```

Penicillium chrysogenum strain 215

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Query  30    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  89
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1687  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1746

Query  150   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  198
             |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1747  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  1795
```

Penicillium chrysogenum strain KCTC6052

Appendix 1.4: Sequence alignments for M1 2 isolate with the three closest identification strains

```
Query  23    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  82
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1628  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1687

Query  83    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  142
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1688  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1747

Query  143   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGG  187
             |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1748  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGG  1792
```

Penicillium tardum strain KCTC16051

```
Query  23    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  82
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1617  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1676

Query  83    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  142
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1677  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1736

Query  143   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  191
             |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1737  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  1785
```

Penicillium brevicompactum strain ATCC 16024

```
Query  23    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  82
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1608  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1667

Query  83    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  142
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1668  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1727

Query  143   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATC  190
             ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1728  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATC  1775
```

Eupenicillium crustaceum

Appendix 1.5: Sequence alignments for M1 3 isolate with the three closest identification strains

```
Query  75    TAAGAGGGTTGGAAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAG  134
             || |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  14    TAGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAG  73

Query  135   GAAGTAAAAGTCGTAACAAGGTTTCC  160
             |||||||||||||||||||||||||||
Sbjct  74    GAAGTAAAAGTCGTAACAAGGTTTCC  99
```

Penicillium fimorum strain DTO 159-F1

```
Query  75    TAAGAGGGTTGGAAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAG  134
             || |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  14    TAGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAG  73

Query  135   GAAGTAAAAGTCGTAACAAGGTTTCC  160
             |||||||||||||||||||||||||||
Sbjct  74    GAAGTAAAAGTCGTAACAAGGTTTCC  99
```

Penicillium fimorum strain CBS 140576

```
Query  75   TAAGAGGGTTGGAAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAG  134
            || |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16   TAGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAG  75

Query  135  GAAGTAAAAGTCGTAACAAGGTTTCC  160
            |||||||||||||||||||||||||||
Sbjct  76   GAAGTAAAAGTCGTAACAAGGTTTCC  101
```

Penicillium sp DR1

Appendix 1.6: Sequence alignments for M1 4 isolate with the three closest identification strains

```
Query  63   gggTTGGAACCGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGGAAGT  122
            ||||||| | ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3    GGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGGAAGT  62

Query  123  AAAAGTCGTAACAAGGT  139
            |||||||||||||||||
Sbjct  63   AAAAGTCGTAACAAGGT  79
```

Penicillium sp. 2 BRO-2013

```
Query  59   gggggggTTGGAACCGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  118
            ||| |||||||| | ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  502  GGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  561

Query  119  AAGTAAAAGTCGTAACAAGGT  139
            |||||||||||||||||||||
Sbjct  562  AAGTAAAAGTCGTAACAAGGT  582
```

Uncultured fungus clone T3_II_1a_04

```
Query  57   TTgggggggTTGGAACCGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGA  116
            || || |||||||| | ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  41   TTAGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGA  100

Query  117  GGAAGTAAAAGTCGTAACAAGGT  139
            |||||||||||||||||||||||
Sbjct  101  GGAAGTAAAAGTCGTAACAAGGT  123
```

Penicillium sp HT-M15-LS

Appendix 1.7: Sequence alignments for M1 8 isolate with the three closest identification strains

```
Query  2     GCTACTACCGATTGNATGGCTCAGTGAGGCCTTGGGATTGGCTTAGGAGGGTTGGCAACG  61
             ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1599  GCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTTAGGAGGGTTGGCAACG  1658

Query  62    ACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAAC  121
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1659  ACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAAC  1718

Query  122   AAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  154
             |||||||||||||||||||||| |||||||||
Sbjct  1719  AAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA  1751
```

Penicillium roqueforti ATCC 10110

```
Query  2     GCTACTACCGATTGNATGGCTCAGTGAGGCCTTGGGATTGGCTTAGGAGGGTTGGCAACG  61
             ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1603  GCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTTAGGAGGGTTGGCAACG  1662

Query  62    ACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAAC  121
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1663  ACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAAC  1722

Query  122   AAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  154
             |||||||||||||||||||||| |||||||||
Sbjct  1723  AAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA  1755
```

Penicillium camemberti strain ATCC 10387

```
Query  2    GCTACTACCGATTGNATGGCTCAGTGAGGCCTTGGGATTGGCTTAGGAGGGTTGGCAACG  61
            ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  34   GCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTTAGGAGGGTTGGCAACG  93

Query  62   ACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAAC  121
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  94   ACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAAC  153

Query  122  AAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  154
            |||||||||||||||||||||||| ||||||||||
Sbjct  154  AAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA  186
```

Penicillium dipodomyicola strain ACBF 002-3

Appendix 1.8: Sequence alignments for M1 10 isolate with the three closest identification strains

```
Query  38    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  97
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1628  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1687

Query  98    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  157
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1688  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1747

Query  158   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGG  202
            |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1748  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGG  1792
```

Penicillium tardum strain KCTC16051

```
Query  38    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  97
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1627  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1686

Query  98    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  157
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1687  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1746

Query  158   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  206
            ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1747  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  1795
```

Penicillium chrysogenum KCTC6052

```
Query  38    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  97
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1609  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1668

Query  98    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  157
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1669  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1728

Query  158   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGC  196
            |||||||||||||||||||||||||||||||||||||||
Sbjct  1729  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGC  1767
```

Penicillium expansum

Appendix 1.9: Sequence alignments for M1 11 isolate with the three closest identification strains

```
Query  23    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  82
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  455   GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  514

Query  83    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  142
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  515   AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  574

Query  143   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGC  181
             |||||||||||||||||||||||||||||||||||||||
Sbjct  575   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGC  613
```

Penicillium chrysogenum strain 215

```
Query  23    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  82
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1608  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1667

Query  83    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  142
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1668  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1727

Query  143   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATC  190
             ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1728  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATC  1775
```

Eupenicillium crustaceum

```
Query  23    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  82
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1617  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1676

Query  83    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  142
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1677  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1736

Query  143   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  191
             |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1737  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  1785
```

Penicillium brevicompactum strain ATCC 16024

Appendix 1.10: Sequence alignments for M1 13 isolate with the three closest identification strains

```
Query  35    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  94
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1617  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1676

Query  95    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  154
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1677  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1736

Query  155   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  203
             |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1737  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  1785
```

Penicillium brevicompactum ATCC 16024

```
Query  35    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  94
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1627  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1686

Query  95    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  154
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1687  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1746

Query  155   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  203
             |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1747  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  1795
```

Penicillium chrysogenum strain KCTC6052

```
Query  35    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  94
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1608  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1667

Query  95    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  154
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1668  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1727

Query  155   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATC  202
             |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1728  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATC  1775
```

Eupenicillium crustaceum

Appendix 1.11: Sequence alignments for M1 14 isolate with the three closest identification strains

```
Query  35    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  94
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1609  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1668

Query  95    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  154
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1669  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1728

Query  155   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGC  193
             |||||||||||||||||||||||||||||||||||||||
Sbjct  1729  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGC  1767
```

Penicillium expansum

```
Query  35    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  94
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  455   GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  514

Query  95    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  154
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  515   AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  574

Query  155   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGC  193
             |||||||||||||||||||||||||||||||||||||||
Sbjct  575   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGC  613
```

Penicillium chrysogenum strain 215

```
Query  35    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  94
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1628  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1687

Query  95    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  154
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1688  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1747

Query  155   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGG  199
             |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1748  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGG  1792
```

Penicillium tardum KCTC16051

Appendix 1.12: Sequence alignments for M1 15 isolate with the three closest identification strains

```
Query  38    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  97
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1627  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1686

Query  98    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  157
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1687  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1746

Query  158   AAGTAAAAGTNGTAANAAGGTT  179
             |||||||||| |||| ||||||
Sbjct  1747  AAGTAAAAGTCGTAACAAGGTT  1768
```

Penicillium solitum strain 20-01

```
Query  38    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  97
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1613  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1672

Query  98    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  157
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1673  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1732

Query  158   AAGTAAAAGTNGTAANAAGGTT  179
             |||||||||| |||| ||||||
Sbjct  1733  AAGTAAAAGTCGTAACAAGGTT  1754
```

Penicillium sp clone NJ-F4 18S

```
Query  38    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  97
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  18    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  77

Query  98    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  157
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  78    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  137

Query  158   AAGTAAAAGTNGTAANAAGGTT  179
             |||||||||| |||| ||||||
Sbjct  138   AAGTAAAAGTCGTAACAAGGTT  159
```

Penicillium dipodomyicola strain ACBF 002-3

Appendix 1.13: Sequence alignments for M1 16 isolate with the three closest identification strains

```
Query  37    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  96
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1583  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1642

Query  97    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  156
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1643  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1702

Query  157   AAGTAAAAGTCGTAACAAGGT  177
             |||||||||||||||||||||
Sbjct  1703  AAGTAAAAGTCGTAACAAGGT  1723
```

Penicillium roqueforti strain ATCC 10110

```
Query  37    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  96
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  10    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  69

Query  97    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  156
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  70    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  129

Query  157   AAGTAAAAGTCGTAACAAGGT  177
             |||||||||||||||||||||
Sbjct  130   AAGTAAAAGTCGTAACAAGGT  150
```

Penicillium sp BF79

```
Query  37    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  96
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  20    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  79

Query  97    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  156
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  80    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  139

Query  157   AAGTAAAAGTCGTAACAAGGT  177
             |||||||||||||||||||||
Sbjct  140   AAGTAAAAGTCGTAACAAGGT  160
```

Penicillium sp BF18

Appendix 1.14: Sequence alignments for M1 18 isolate with the three closest identification strains

```
Query  37   GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  96
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  123  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  182

Query  97   AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  156
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  183  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  242

Query  157  AAGTAAAAGTCGTAACAAGGTTTCC  181
            |||||||||||||||||||||||||
Sbjct  243  AAGTAAAAGTCGTAACAAGGTTTCC  267
```

Penicillium sclerotigenum strain NRRL 22813

```
Query  37   GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  96
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  130  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  189

Query  97   AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  156
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  190  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  249

Query  157  AAGTAAAAGTCGTAACAAGGTTTCC  181
            |||||||||||||||||||||||||
Sbjct  250  AAGTAAAAGTCGTAACAAGGTTTCC  274
```

*Penicilliumvulpinumstrain*NRRL1002

```
Query  37   GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  96
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  131  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  190

Query  97   AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  156
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  191  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  250

Query  157  AAGTAAAAGTCGTAACAAGGTTTCC  181
            |||||||||||||||||||||||||
Sbjct  251  AAGTAAAAGTCGTAACAAGGTTTCC  275
```

Penicillium vulpinum strain NRRL 2031

Appendix 1.15: Sequence alignments for M1 20 isolate with the three closest identification strains

```
Query  3    GCTACTACCGATTGNATGGCTCAGTGAGGCCTTGGGATTGGCTTAGGAGGGTTGGCAACG  62
            |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  167  GCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTTAGGAGGGTTGGCAACG  226

Query  63   ACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAAC  122
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  227  ACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAAC  286

Query  123  AAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  155
            ||||||||||||||||||||||| |||||||||
Sbjct  287  AAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA  319
```

Penicillium clavigerum NRRL 1003

```
Query  3    GCTACTACCGATTGNATGGCTCAGTGAGGCCTTGGGATTGGCTTAGGAGGGTTGGCAACG  62
            |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  168  GCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTTAGGAGGGTTGGCAACG  227

Query  63   ACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAAC  122
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  228  ACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAAC  287

Query  123  AAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  155
            ||||||||||||||||||||||| |||||||||
Sbjct  288  AAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA  320
```

Penicillium griseofulvum NRRL 3523

```
Query  3     GCTACTACCGATTGNATGGCTCAGTGAGGCCTTGGGATTGGCTTAGGAGGGTTGGCAACG  62
             ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  178   GCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTTAGGAGGGTTGGCAACG  237

Query  63    ACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAAC  122
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  238   ACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAAC  297

Query  123   AAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  155
             |||||||||||||||||||||||| |||||||||
Sbjct  298   AAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA  330
```

Penicillium expansum strain NRRL 35259

Appendix 1.16: Sequence alignments for M1 22 isolate with the three closest identification strains

```
Query  38    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  97
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1617  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1676

Query  98    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  157
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1677  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1736

Query  158   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  206
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1737  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  1785
```

Penicillium brevicompactum ATCC 16024

```
Query  38    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  97
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1627  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1686

Query  98    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  157
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1687  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1746

Query  158   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  206
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1747  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  1795
```

Penicillium chrysogenum strain KCTC6052

```
Query  38    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  97
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1609  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1668

Query  98    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  157
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1669  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1728

Query  158   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGC  196
             |||||||||||||||||||||||||||||||||||||||
Sbjct  1729  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGC  1767
```

Penicillium expansum

Appendix 1.17: Sequence alignments for Es1 isolate with the three closest identification strains

```
Query  25    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  84
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1617  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1676

Query  85    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  144
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1677  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1736

Query  145   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  193
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1737  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  1785
```

Penicillium brevicompactum strain ATCC 16024

```
Query  25    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  84
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1627  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1686

Query  85    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  144
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1687  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1746

Query  145   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  193
             |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1747  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  1795
```

Penicillium chrysogenum strain KCTC6052

```
Query  25    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  84
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1608  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1667

Query  85    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  144
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1668  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1727

Query  145   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATC  192
             ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1728  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATC  1775
```

Eupenicillium crustaceum

Appendix 1.18: Sequence alignments for Es2 isolate with the three closest identification strains

```
Query  29    GTACACCCCTCCCGTCGTTACTACCGATTGAATGGGTCAGTGAGGCCTCGGGATTGGATT  88
             ||||||  ||  |||||||  ||||||||||||||||||  ||||||||||||  ||||||||  ||
Sbjct  1617  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1676

Query  89    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  148
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1677  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1736

Query  149   AAGTAAAAGTCGTAACAAGGTTTC  172
             ||||||||||||||||||||||||
Sbjct  1737  AAGTAAAAGTCGTAACAAGGTTTC  1760
```

Penicillium brevicompactum strain ATCC 16024

```
Query  29    GTACACCCCTCCCGTCGTTACTACCGATTGAATGGGTCAGTGAGGCCTCGGGATTGGATT  88
             ||||||  ||  |||||||  ||||||||||||||||||  ||||||||||||  ||||||||  ||
Sbjct  127   GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  186

Query  89    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  148
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  187   AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  246

Query  149   AAGTAAAAGTCGTAACAAGGTTTC  172
             ||||||||||||||||||||||||
Sbjct  247   AAGTAAAAGTCGTAACAAGGTTTC  270
```

Penicillium chrysogenum strain 41H1

```
Query  29    GTACACCCCTCCCGTCGTTACTACCGATTGAATGGGTCAGTGAGGCCTCGGGATTGGATT  88
             ||||||  ||  |||||||  ||||||||||||||||||  ||||||||||||  ||||||||  ||
Sbjct  70    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  129

Query  89    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  148
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  130   AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  189

Query  149   AAGTAAAAGTCGTAACAAGGTTTC  172
             ||||||||||||||||||||||||
Sbjct  190   AAGTAAAAGTCGTAACAAGGTTTC  213
```

Uncultured fungus clone CMH605

Appendix 1.19: Sequence alignments for Es9 isolate with the three closest identification strains

```
Query  23    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  82
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  455   GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  514

Query  83    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  142
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  515   AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  574

Query  143   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGC  181
             |||||||||||||||||||||||||||||||||||||||
Sbjct  575   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGC  613
```

Penicillium chrysogenum strain 215

```
Query  23    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  82
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1609  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1668

Query  83    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  142
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1669  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1728

Query  143   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGC  181
             |||||||||||||||||||||||||||||||||||||||
Sbjct  1729  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGC  1767
```

Penicillium expansum

```
Query  23    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  82
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1627  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1686

Query  83    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  142
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1687  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1746

Query  143   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  191
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1747  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  1795
```

Penicillium chrysogenum strain KCTC6052

Appendix 1.20: Sequence alignments for Ant w1 isolate with the three closest identification strains

```
Query  1     ACTACCGATTGAATGGCTCAGTGAGGCGTCCGGACTGGCCCAGGGAGGTGGGCAACTACC  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  209   ACTACCGATTGAATGGCTCAGTGAGGCGTCCGGACTGGCCCAGGGAGGTGGGCAACTACC  268

Query  61    ACCCAGGGCCGGAAAGCTCTCCAAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAACAAG  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  269   ACCCAGGGCCGGAAAGCTCTCCAAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAACAAG  328

Query  121   GTCTCCGTAGGTGAACCTGC  140
             ||||||||||||||||||||
Sbjct  329   GTCTCCGTAGGTGAACCTGC  348
```

Verticillium fungicola solate ARSEF 2065

```
Query  1     ACTACCGATTGAATGGCTCAGTGAGGCGTCCGGACTGGCCCAGGGAGGTGGGCAACTACC  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  209   ACTACCGATTGAATGGCTCAGTGAGGCGTCCGGACTGGCCCAGGGAGGTGGGCAACTACC  268

Query  61    ACCCAGGGCCGGAAAGCTCTCCAAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAACAAG  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  269   ACCCAGGGCCGGAAAGCTCTCCAAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAACAAG  328

Query  121   GTCTCCGTAGGTGAACCTGC  140
             ||||||||||||||||||||
Sbjct  329   GTCTCCGTAGGTGAACCTGC  348
```

Simplicillium lamellicola isolate UAMH 2055

```
Query  1    ACTACCGATTGAATGGCTCAGTGAGGCGTCCGGACTGGCCCAGGGAGGTGGGCAACTACC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  208  ACTACCGATTGAATGGCTCAGTGAGGCGTCCGGACTGGCCCAGGGAGGTGGGCAACTACC  267

Query  61   ACCCAGGGCCGGAAAGCTCTCCAAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAACAAG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  268  ACCCAGGGCCGGAAAGCTCTCCAAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAACAAG  327

Query  121  GTCTCCGTAGGTGAACCTGC  140
            ||||||||||||||||||||
Sbjct  328  GTCTCCGTAGGTGAACCTGC  347
```

Phytocordyceps ninchukispora strain CCRC 31900

Appendix 1.21: Sequence alignments for Ant w2 isolate with the three closest identification strains

```
Query  3     CTACTACCGATTGAATGGCTCAGTGAGGCGTCCGGACTGGCCCAGGGAGGTGGG-AT-TA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||| |   ||
Sbjct  1558  CTACTACCGATTGAATGGCTCAGTGAGGCGTCCGGACTGGCCCAGGGAGGTGGGCAACTA  1617

Query  61    CCACCCCTGGGCGGAAATCTCTCCCCCCTCGGGCATTT  98
             ||||||   ||  ||||||  ||||||    |||||  |||||
Sbjct  1618  CCACCCAGGGCCGGAAAGCTCTCCAAACTCGGTCATTT  1655
```

Lecanicillium sp strain CCF 5201

```
Query  3     CTACTACCGATTGAATGGCTCAGTGAGGCGTCCGGACTGGCCCAGGGAGGTGGG-AT-TA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||| |   ||
Sbjct  1558  CTACTACCGATTGAATGGCTCAGTGAGGCGTCCGGACTGGCCCAGGGAGGTGGGCAACTA  1617

Query  61    CCACCCCTGGGCGGAAATCTCTCCCCCCTCGGGCATTT  98
             ||||||   ||  ||||||  ||||||    |||||  |||||
Sbjct  1618  CCACCCAGGGCCGGAAAGCTCTCCAAACTCGGTCATTT  1655
```

Lecanicillium sp CCF 5233

```
Query  3     CTACTACCGATTGAATGGCTCAGTGAGGCGTCCGGACTGGCCCAGGGAGGTGGG-AT-TA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||| |   ||
Sbjct  1624  CTACTACCGATTGAATGGCTCAGTGAGGCGTCCGGACTGGCCCAGGGAGGTGGGCAACTA  1683

Query  61    CCACCCCTGGGCGGAAATCTCTCCCCCCTCGGGCATTT  98
             ||||||   ||  ||||||  ||||||    |||||  |||||
Sbjct  1684  CCACCCAGGGCCGGAAAGCTCTCCAAACTCGGTCATTT  1721
```

Fungal sp. SLZ14

Appendix 1.22: Sequence alignments for Ant w3 isolate with the three closest identification strains

```
Query  37   GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCGTCCGGACTGGCCC  96
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  190  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCGTCCGGACTGGCCC  249

Query  97   AGGGAGGTGGGCAACTACCACCCAGGGCCGGAAAGCTCTCCAAACTCGGTCATTTAGAGG  156
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  250  AGGGAGGTGGGCAACTACCACCCAGGGCCGGAAAGCTCTCCAAACTCGGTCATTTAGAGG  309

Query  157  AAGTAAAAGTCGTAACAAGGTCTCCGTAGGTGAACCTGC  195
            |||||||||||||||||||||||||||||||||||||||
Sbjct  310  AAGTAAAAGTCGTAACAAGGTCTCCGTAGGTGAACCTGC  348
```

Simplicillium lamellicola isolate UAMH 2055

```
Query  37    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCGTCCGGACTGGCCC  96
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  190   GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCGTCCGGACTGGCCC  249

Query  97    AGGGAGGTGGGCAACTACCACCCAGGGCCGGAAAGCTCTCCAAACTCGGTCATTTAGAGG  156
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  250   AGGGAGGTGGGCAACTACCACCCAGGGCCGGAAAGCTCTCCAAACTCGGTCATTTAGAGG  309

Query  157   AAGTAAAAGTCGTAACAAGGTCTCCGTAGGTGAACCTGC  195
             |||||||||||||||||||||||||||||||||||||||
Sbjct  310   AAGTAAAAGTCGTAACAAGGTCTCCGTAGGTGAACCTGC  348
```

Cordyceps sp ATCC 36337

```
Query  37    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCGTCCGGACTGGCCC  96
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1607  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCGTCCGGACTGGCCC  1666

Query  97    AGGGAGGTGGGCAACTACCACCCAGGGCCGGAAAGCTCTCCAAACTCGGTCATTTAGAGG  156
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1667  AGGGAGGTGGGCAACTACCACCCAGGGCCGGAAAGCTCTCCAAACTCGGTCATTTAGAGG  1726

Query  157   AAGTAAAAGTCGTAACAAGGTCTCCGTAGGTGAACCTGC  195
             |||||||||||||||||||||||||||||||||||||||
Sbjct  1727  AAGTAAAAGTCGTAACAAGGTCTCCGTAGGTGAACCTGC  1765
```

Fungal sp SLZ46

Appendix 1.23: Sequence alignments for Ant b4 isolate with the three closest identification strains

```
Query  36    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTAAGTGAGGCCTCGGGACTGGCTC  95
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1614  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTAAGTGAGGCCTCGGGACTGGCTC  1673

Query  96    AGAGAGGTCGGCAACGACCACTCAGAGCTGGAAACTTGTTCAAACTTGGTCATTTAGAGG  155
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1674  AGAGAGGTCGGCAACGACCACTCAGAGCTGGAAACTTGTTCAAACTTGGTCATTTAGAGG  1733

Query  156   AAGTAAAAGTCGTAACAAGGTCTTCGTAG  184
             |||||||||||||||||||||||||||||
Sbjct  1734  AAGTAAAAGTCGTAACAAGGTCTTCGTAG  1762
```

Uncultured marine ascomycete clone PRTBE7359

```
Query  36    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTAAGTGAGGCCTCGGGACTGGCTC  95
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1476  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTAAGTGAGGCCTCGGGACTGGCTC  1535

Query  96    AGAGAGGTCGGCAACGACCACTCAGAGCTGGAAACTTGTTCAAACTTGGTCATTTAGAGG  155
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1536  AGAGAGGTCGGCAACGACCACTCAGAGCTGGAAACTTGTTCAAACTTGGTCATTTAGAGG  1595

Query  156   AAGTAAAAGTCGTAACAAGGTCTTCGTA  183
             ||||||||||||||||||||||||||||
Sbjct  1596  AAGTAAAAGTCGTAACAAGGTCTTCGTA  1623
```

Uncultured marine ascomycete clone PRTBE7237

```
Query  36    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTAAGTGAGGCCTCGGGACTGGCTC  95
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1583  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTAAGTGAGGCCTCGGGACTGGCTC  1642

Query  96    AGAGAGGTCGGCAACGACCACTCAGAGCTGGAAACTTGTTCAAACTTGGTCATTTAGAGG  155
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1643  AGAGAGGTCGGCAACGACCACTCAGAGCTGGAAACTTGTTCAAACTTGGTCATTTAGAGG  1702

Query  156   AAGTAAAAGTCGTAACAAGGTCTTCGTAGGTGAACCTGCAGAAGGATCA  204
             |||||||||||||||||||||||||||||||||||||| || |||||||
Sbjct  1703  AAGTAAAAGTCGTAACAAGGTCTTCGTAGGTGAACCTGCGGAGGGATCA  1751
```

Exophiala oligosperma strain CBS 725.88

Appendix 1.24: Sequence alignments for M35 1 isolate with the three closest identification strains

```
Query  36    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  95
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1583  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1642

Query  96    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  155
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1643  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1702

Query  156   AAGTAAAAGTCGTAACAAGGTTTCC  180
             |||||||||||||||||||||||||
Sbjct  1703  AAGTAAAAGTCGTAACAAGGTTTCC  1727
```

Penicillium roqueforti strain ATCC 10110

```
Query  36    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  95
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  18    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  77

Query  96    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  155
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  78    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  137

Query  156   AAGTAAAAGTCGTAACAAGGTTTCC  180
             |||||||||||||||||||||||||
Sbjct  138   AAGTAAAAGTCGTAACAAGGTTTCC  162
```

Penicillium dipodomyicola strain ACBF 002-3

```
Query  36    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  95
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  10    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  69

Query  96    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  155
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  70    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  129

Query  156   AAGTAAAAGTCGTAACAAGGTTTCC  180
             |||||||||||||||||||||||||
Sbjct  130   AAGTAAAAGTCGTAACAAGGTTTCC  154
```

Penicillium sp BF79

Appendix 1.25: Sequence alignments for M35 4 isolate with the three closest identification strains

```
Query  2     GCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTTAGGAGGGTTGGCAACG  61
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  471   GCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTTAGGAGGGTTGGCAACG  530

Query  62    ACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAAC  121
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  531   ACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAAC  590

Query  122   AAGGTTTCCGTAGGTGAACCTGC  144
             |||||||||||||||||||||||
Sbjct  591   AAGGTTTCCGTAGGTGAACCTGC  613
```

Penicillium chrysogenum strain 215

```
Query  2     GCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTTAGGAGGGTTGGCAACG  61
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1625  GCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTTAGGAGGGTTGGCAACG  1684

Query  62    ACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAAC  121
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1685  ACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAAC  1744

Query  122   AAGGTTTCCGTAGGTGAACCTGC  144
             |||||||||||||||||||||||
Sbjct  1745  AAGGTTTCCGTAGGTGAACCTGC  1767
```

Penicillium expansum

```
Query  2     GCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTTAGGAGGGTTGGCAACG  61
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1644  GCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTTAGGAGGGTTGGCAACG  1703

Query  62    ACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAAC  121
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1704  ACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAAC  1763

Query  122   AAGGTTTCCGTAGGTGAACCTGCAGAAGG  150
             |||||||||||||||||||||||||||||
Sbjct  1764  AAGGTTTCCGTAGGTGAACCTGCAGAAGG  1792
```

Penicillium tardum KCTC16051

Appendix 1.26: Sequence alignments for M35 5 isolate with the three closest identification strains

```
Query  38    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  97
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1617  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1676

Query  98    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  157
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1677  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1736

Query  158   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  206
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1737  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  1785
```

Penicillium brevicompactum strain ATCC 16024

```
Query  38    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  97
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1627  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1686

Query  98    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  157
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1687  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1746

Query  158   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  206
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1747  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  1795
```

Penicillium chrysogenum KCTC6052

```
Query  38    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  97
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1608  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1667

Query  98    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  157
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1668  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1727

Query  158   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATC  205
             |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1728  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATC  1775
```

Eupenicillium crustaceum

Appendix 1.27: Sequence alignments for M35 9 isolate with the three closest identification strains

```
Query  38    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  97
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  123   GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  182

Query  98    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  157
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  183   AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  242

Query  158   AAGTAAAAGTCGTAACAAGGTT  179
             ||||||||||||||||||||||
Sbjct  243   AAGTAAAAGTCGTAACAAGGTT  264
```

Penicillium glandicola NRRL 985

```
Query  38   GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  97
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  130  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  189

Query  98   AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  157
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  190  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  249

Query  158  AAGTAAAAGTCGTAACAAGGTT  179
            ||||||||||||||||||||||
Sbjct  250  AAGTAAAAGTCGTAACAAGGTT  271
```

Penicillium vulpinum strain NRRL 1002

```
Query  38   GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  97
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  134  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  193

Query  98   AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  157
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  194  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  253

Query  158  AAGTAAAAGTCGTAACAAGGTT  179
            ||||||||||||||||||||||
Sbjct  254  AAGTAAAAGTCGTAACAAGGTT  275
```

Penicillium glandicola strain NRRL 2036

Appendix 1.28: Sequence alignments for M35 11 isolate with the three closest identification strains

```
Query  35   GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  94
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  70   GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  129

Query  95   AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  154
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  130  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  189

Query  155  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA  203
            ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  190  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA  238
```

Penicillium sp HT-M15-L

```
Query  44   GCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTTAGGAGGGTT  103
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    GCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTTAGGAGGGTT  60

Query  104  GGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGGAAGTAAAAG  163
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   GGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGGAAGTAAAAG  120

Query  164  TCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA  203
            ||||||||||||||||||||||||||||||||||||||||
Sbjct  121  TCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA  160
```

Uncultured fungus clone CMH605

```
Query  35   GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  94
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  70   GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  129

Query  95   AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  154
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  130  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  189

Query  155  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA  203
            ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  190  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA  238
```

Uncultured fungus clone CMH224

Appendix 1.29: Sequence alignments for Pp3 isolate with the three closest identification strains

```
Query  38    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  97
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1591  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1650

Query  98    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  157
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1651  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1710

Query  158   AAGTAAAAGTCAGTAACAAGGTT-CCGTAGGTAACCCTGCGG  198
             |||||||||||| |||||||||| |||||||| | |||||||
Sbjct  1711  AAGTAAAAGTC-GTAACAAGGTTTCCGTAGGTGAACCTGCGG  1751
```

Penicillium commune strain MA09-AL

```
Query  38    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  97
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  11    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  70

Query  98    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  157
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  71    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  130

Query  158   AAGTAAAAGTCAGTAACAAGGTT-CCGTAGGTAACCCTGCGG  198
             |||||||||||| |||||||||| |||||||| | |||||||
Sbjct  131   AAGTAAAAGTC-GTAACAAGGTTTCCGTAGGTGAACCTGCGG  171
```

Penicillium coccotrypicola strain BRIP 59608

```
Query  38    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  97
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  123   GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  182

Query  98    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  157
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  183   AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  242

Query  158   AAGTAAAAGTCAGTAACAAGGTT-CCGTAGGTAACCCTGCGG  198
             |||||||||||| |||||||||| |||||||| | |||||||
Sbjct  243   AAGTAAAAGTC-GTAACAAGGTTTCCGTAGGTGAACCTGCGG  283
```

Penicillium glandicola strain NRRL 985

Appendix 1.30: Sequence alignments for S5 isolate with the three closest identification strains

```
Query  37    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGACTGGCTC  96
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1625  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGACTGGCTC  1684

Query  97    AGGAGGGTTGGCAACGACCCCCCAGAGCCGGAAACTTGGTCAAACTCGGTCATTTAGAGG  156
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1685  AGGAGGGTTGGCAACGACCCCCCAGAGCCGGAAACTTGGTCAAACTCGGTCATTTAGAGG  1744

Query  157   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  205
             |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1745  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  1793
```

Uncultured *Penicillium* clone USN5

```
Query  37    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGACTGGCTC  96
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1625  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGACTGGCTC  1684

Query  97    AGGAGGGTTGGCAACGACCCCCCAGAGCCGGAAACTTGGTCAAACTCGGTCATTTAGAGG  156
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1685  AGGAGGGTTGGCAACGACCCCCCAGAGCCGGAAACTTGGTCAAACTCGGTCATTTAGAGG  1744

Query  157   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  205
             |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1745  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  1793
```

Penicillium sp strain Y12 EG-2010

```
Query  37    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGACTGGCTC  96
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1627  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGACTGGCTC  1686

Query  97    AGGAGGGTTGGCAACGACCCCCCAGAGCCGGAAACTTGGTCAAACTCGGTCATTTAGAGG  156
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1687  AGGAGGGTTGGCAACGACCCCCCAGAGCCGGAAACTTGGTCAAACTCGGTCATTTAGAGG  1746

Query  157   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  205
             |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1747  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  1795
```

Penicillium glabrum strain KCTC16099

Appendix 1.31: Sequence alignments for S6 isolate with the three closest identification strains

```
Query  38    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCGTCCGGACTGGCCC  97
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1554  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCGTCCGGACTGGCCC  1613

Query  98    AGAGAGGTGGGCAACTACCACTCAGGGCCGGAAAGCTCTCCAAACTCGGTCATTTAGAGG  157
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1614  AGAGAGGTGGGCAACTACCACTCAGGGCCGGAAAGCTCTCCAAACTCGGTCATTTAGAGG  1673

Query  158   AAGTAAAAGTCGTAACAAGGTCTCCGTAGGTGAACCTGC  196
             |||||||||||||||||||||||||||||||||||||||
Sbjct  1674  AAGTAAAAGTCGTAACAAGGTCTCCGTAGGTGAACCTGC  1712
```

Fungal sp Isolate nussu_195

```
Query  38    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCGTCCGGACTGGCCC  97
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1607  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCGTCCGGACTGGCCC  1666

Query  98    AGAGAGGTGGGCAACTACCACTCAGGGCCGGAAAGCTCTCCAAACTCGGTCATTTAGAGG  157
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1667  AGAGAGGTGGGCAACTACCACTCAGGGCCGGAAAGCTCTCCAAACTCGGTCATTTAGAGG  1726

Query  158   AAGTAAAAGTCGTAACAAGGTCTCCGTAGGTGAACCTGC  196
             |||||||||||||||||||||||||||||||||||||||
Sbjct  1727  AAGTAAAAGTCGTAACAAGGTCTCCGTAGGTGAACCTGC  1765
```

Fungal sp SLZ62

```
Query  38    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCGTCCGGACTGGCCC  97
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1609  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCGTCCGGACTGGCCC  1668

Query  98    AGAGAGGTGGGCAACTACCACTCAGGGCCGGAAAGCTCTCCAAACTCGGTCATTTAGAGG  157
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1669  AGAGAGGTGGGCAACTACCACTCAGGGCCGGAAAGCTCTCCAAACTCGGTCATTTAGAGG  1728

Query  158   AAGTAAAAGTCGTAACAAGGTCTCCGTAGGTGAACCTGC  196
             |||||||||||||||||||||||||||||||||||||||
Sbjct  1729  AAGTAAAAGTCGTAACAAGGTCTCCGTAGGTGAACCTGC  1767
```

Hypocrea sp SP-4

Appendix 1.32: Sequence alignments for Sa1 isolate with the three closest identification strains

```
Query  38    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  97
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1609  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1668

Query  98    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  157
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1669  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1728

Query  158   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGC  196
             |||||||||||||||||||||||||||||||||||||||
Sbjct  1729  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGC  1767
```

Penicillium expansum

```
Query  38    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  97
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  455   GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  514

Query  98    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  157
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  515   AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  574

Query  158   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGC  196
             ||||||||||||||||||||||||||||||||||||||||
Sbjct  575   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGC  613
```

Penicillium chrysogenum strain 215

```
Query  38    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  97
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1608  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1667

Query  98    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  157
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1668  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1727

Query  158   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATC  205
             ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1728  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATC  1775
```

Eupenicillium crustaceum

Appendix 1.33: Sequence alignments for Sa2 isolate with the three closest identification strains

```
Query  38    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  97
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  190   GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  249

Query  98    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  157
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  250   AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  309

Query  158   AAGTAAAAGTCGTAACAAGGTTTCC  182
             |||||||||||||||||||||||||
Sbjct  310   AAGTAAAAGTCGTAACAAGGTTTCC  334
```

Trichocomaceae sp. LM196

```
Query  38    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  97
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1531  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1590

Query  98    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  157
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1591  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1650

Query  158   AAGTAAAAGTCGTAACAAGGTT  179
             ||||||||||||||||||||||
Sbjct  1651  AAGTAAAAGTCGTAACAAGGTT  1672
```

Uncultured *ascomycete*

```
Query  38    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  97
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481   GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  540

Query  98    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  157
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541   AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  600

Query  158   AAGTAAAAGTCGTAACAAGGTTTCC  182
             |||||||||||||||||||||||||
Sbjct  601   AAGTAAAAGTCGTAACAAGGTTTCC  625
```

Penicillium griseofulvum

Appendix 1.34: Sequence alignments for W1 isolate with the three closest identification strains

```
Query  2     GCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTTAGGAGGGTTGGCAACG  61
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1645  GCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTTAGGAGGGTTGGCAACG  1704

Query  62    ACCCCCCCGGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAAC  121
             |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1705  ACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAAC  1764

Query  122   AAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA  154
             ||||||||||||||||||||||||||||||||
Sbjct  1765  AAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA  1797
```

Penicillium freii (IBT 3464)

```
Query  2     GCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTTAGGAGGGTTGGCAACG  61
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  36    GCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTTAGGAGGGTTGGCAACG  95

Query  62    ACCCCCCCGGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAAC  121
             ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  96    ACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAAC  155

Query  122   AAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA  154
             ||||||||||||||||||||||||||||||||
Sbjct  156   AAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA  188
```

Penicillium dipodomyicola strain B64

```
Query  2     GCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTTAGGAGGGTTGGCAACG  61
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  150   GCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTTAGGAGGGTTGGCAACG  209

Query  62    ACCCCCCCGGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAAC  121
             ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  210   ACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAAC  269

Query  122   AAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA  154
             ||||||||||||||||||||||||||||||||
Sbjct  270   AAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA  302
```

Penicillium gladioli strain NRRL 939

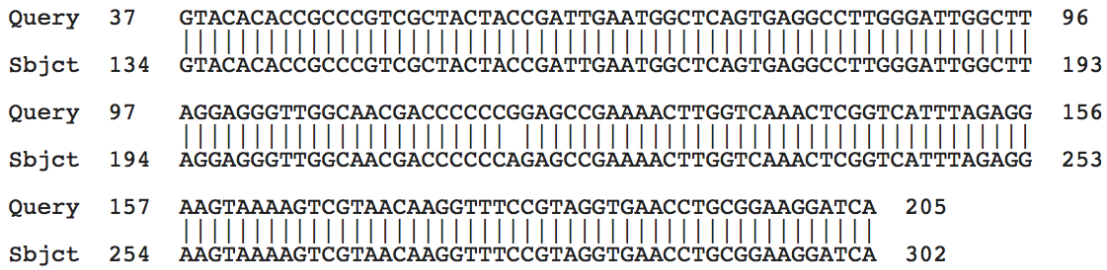
Appendix 1.35: Sequence alignments for W2 isolate with the three closest identification strains

```
Query  37    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  96
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  130   GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  189

Query  97    AGGAGGGTTGGCAACGACCCCCCGGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  156
             |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  190   AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  249

Query  157   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA  205
             |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  250   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA  298
```

Penicillium paneum strain NRRL 25162

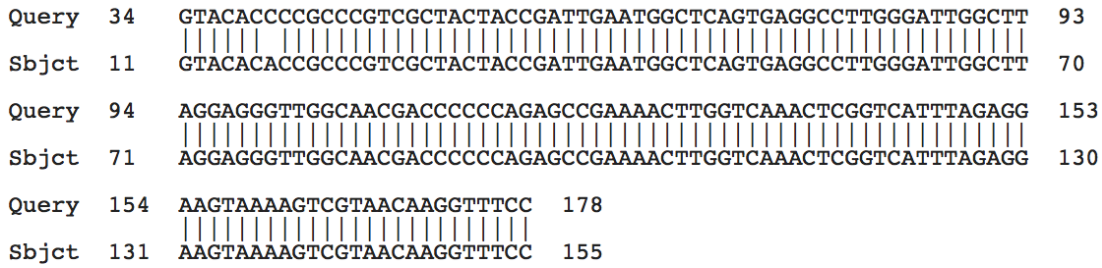
```
Query  37    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  96
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  134   GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  193

Query  97    AGGAGGGTTGGCAACGACCCCCCGGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  156
             |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  194   AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  253

Query  157   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA  205
             |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  254   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA  302
```

Penicillium solitum strain 20-01

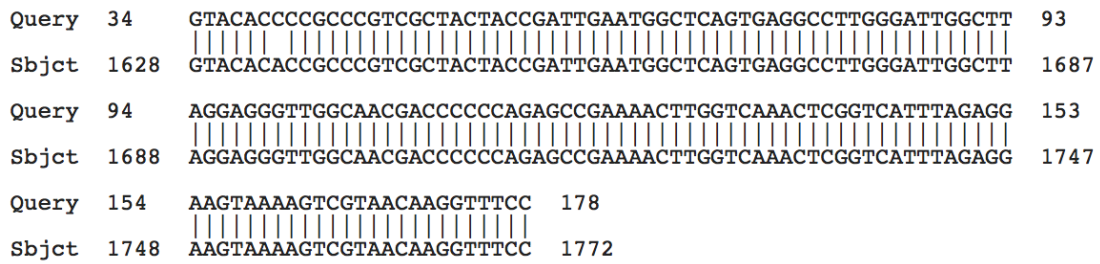


Penicillium echinulatum isolate 00044

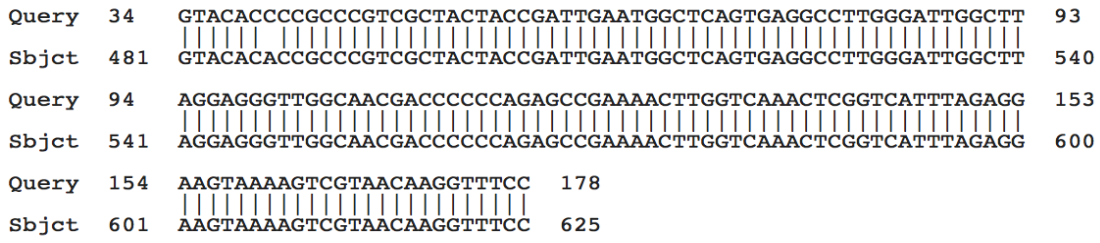
Appendix 1.36: Sequence alignments for W3 isolate with the three closest identification strains



Penicillium coccotrypicola strain BRIP 59608



Penicillium tardum strain KCTC16051



Penicillium griseofulvum

Appendix 1.37: Sequence alignments for W6 isolate with the three closest identification strains

```
Query  22    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  81
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1617  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1676

Query  82    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAAAGG  141
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||
Sbjct  1677  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1736

Query  142   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  190
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1737  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  1785
```

Penicillium brevicompactum strain ATCC 16024

```
Query  22    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  81
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1627  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1686

Query  82    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAAAGG  141
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||
Sbjct  1687  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1746

Query  142   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  190
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1747  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  1795
```

Penicillium chrysogenum strain KCTC6052

```
Query  22    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  81
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1608  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1667

Query  82    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAAAGG  141
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||
Sbjct  1668  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1727

Query  142   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATC  189
             |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1728  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATC  1775
```

Eupenicillium crustaceum

Appendix 1.38: Sequence alignments for W7 isolate with the three closest identification strains

```
Query  38    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  97
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  130   GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  189

Query  98    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  157
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  190   AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  249

Query  158   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGGGAACCTGCGGA  199
             |||||||||||||||||||||||||||||| ||||||||||||
Sbjct  250   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGA  291
```

Penicillium vulpinum NRRL 1002

```
Query  38    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  97
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  130   GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  189

Query  98    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  157
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  190   AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  249

Query  158   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGGGAACCTGCGGA  199
             |||||||||||||||||||||||||||||| ||||||||||||
Sbjct  250   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGA  291
```

Penicillium paneum NRRL 25162

```
Query  38   GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  97
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  134  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  193

Query  98   AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  157
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  194  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  253

Query  158  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGGGAACCTGCGGA  199
            |||||||||||||||||||||||||||||| ||||||||||
Sbjct  254  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGA  295
```

Penicillium gladioli strain NRRL 939

Appendix 1.39: Sequence alignments for W9 isolate with the three closest identification strains

```
Query  37    GTACACCCCGCCCGTCGCTACTACCGATTGAATGGGTCAGTGAGGCCTTGGGATTGGCTT  96
            ||||||  |||||||||||||||||||||||||||  |||||||||||||||||||||||
Sbjct  1613  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1672

Query  97    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  156
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1673  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1732

Query  157   AAGTAAAAGTCGTAACAAGGTTTCC  181
            |||||||||||||||||||||||||
Sbjct  1733  AAGTAAAAGTCGTAACAAGGTTTCC  1757
```

Penicillium sp clone NJ-F4 18S

```
Query  37   GTACACCCCGCCCGTCGCTACTACCGATTGAATGGGTCAGTGAGGCCTTGGGATTGGCTT  96
           ||||||  |||||||||||||||||||||||||||  |||||||||||||||||||||||
Sbjct  70   GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  129

Query  97   AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  156
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  130  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  189

Query  157  AAGTAAAAGTCGTAACAAGGTTTCC  181
           |||||||||||||||||||||||||
Sbjct  190  AAGTAAAAGTCGTAACAAGGTTTCC  214
```

Uncultured fungus clone CMH067

```
Query  37   GTACACCCCGCCCGTCGCTACTACCGATTGAATGGGTCAGTGAGGCCTTGGGATTGGCTT  96
           ||||||  |||||||||||||||||||||||||||  |||||||||||||||||||||||
Sbjct  70   GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  129

Query  97   AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  156
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  130  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  189

Query  157  AAGTAAAAGTCGTAACAAGGTTTCC  181
           |||||||||||||||||||||||||
Sbjct  190  AAGTAAAAGTCGTAACAAGGTTTCC  214
```

Uncultured fungus clone CMH605

Appendix 1.40: Sequence alignments for W12 isolate with the three closest identification strains

```
Query  38    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  97
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1609  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1668

Query  98    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  157
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1669  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1728

Query  158   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGC  196
            |||||||||||||||||||||||||||||||||||||||
Sbjct  1729  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGC  1767
```

Penicillium expansum

```
Query  38    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  97
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  455   GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  514

Query  98    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  157
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  515   AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  574

Query  158   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGC  196
             |||||||||||||||||||||||||||||||||||||||
Sbjct  575   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGC  613
```

Penicillium chrysogenum strain 215

```
Query  38    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  97
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1608  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1667

Query  98    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  157
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1668  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1727

Query  158   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATC  205
             ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1728  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATC  1775
```

Eupenicillium crustaceum

Appendix 1.41: Sequence alignments for W13 isolate with the three closest identification strains

```
Query  37    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  96
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1617  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1676

Query  97    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  156
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1677  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1736

Query  157   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  205
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1737  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  1785
```

Penicillium brevicompactum strain ATCC 16024

```
Query  37    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  96
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1627  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1686

Query  97    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  156
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1687  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1746

Query  157   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  205
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1747  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA  1795
```

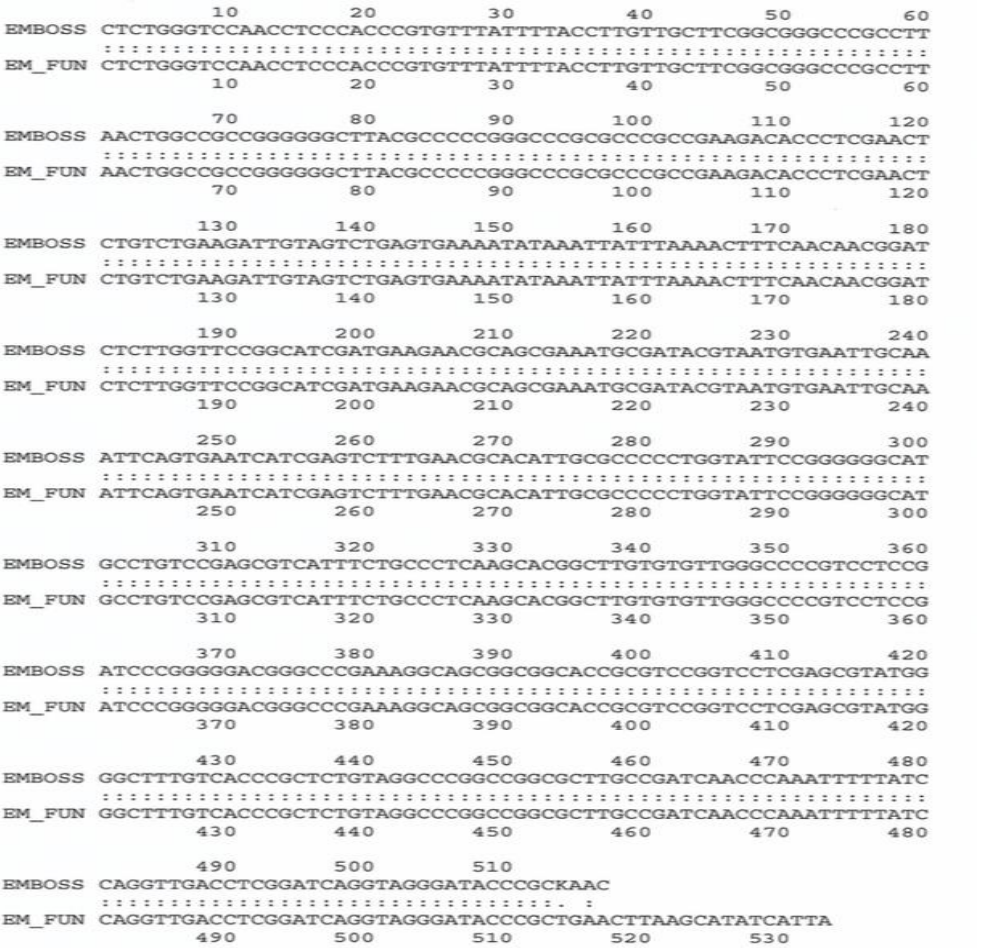
Penicillium chrysogenum strain KCTC6052

```
Query  37    GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  96
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1608  GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCCTTGGGATTGGCTT  1667

Query  97    AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  156
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1668  AGGAGGGTTGGCAACGACCCCCCAGAGCCGAAAACTTGGTCAAACTCGGTCATTTAGAGG  1727

Query  157   AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATC  204
             ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1728  AAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATC  1775
```

Eupenicillium crustaceum

Appendix 1.42: Sequence alignments for Es1 isolate with *Penicillium* sp. (ITS)

Appendix 1.43: Sequence alignments for M 1 11 isolate with *Penicillium chrysogenum* (ITS)



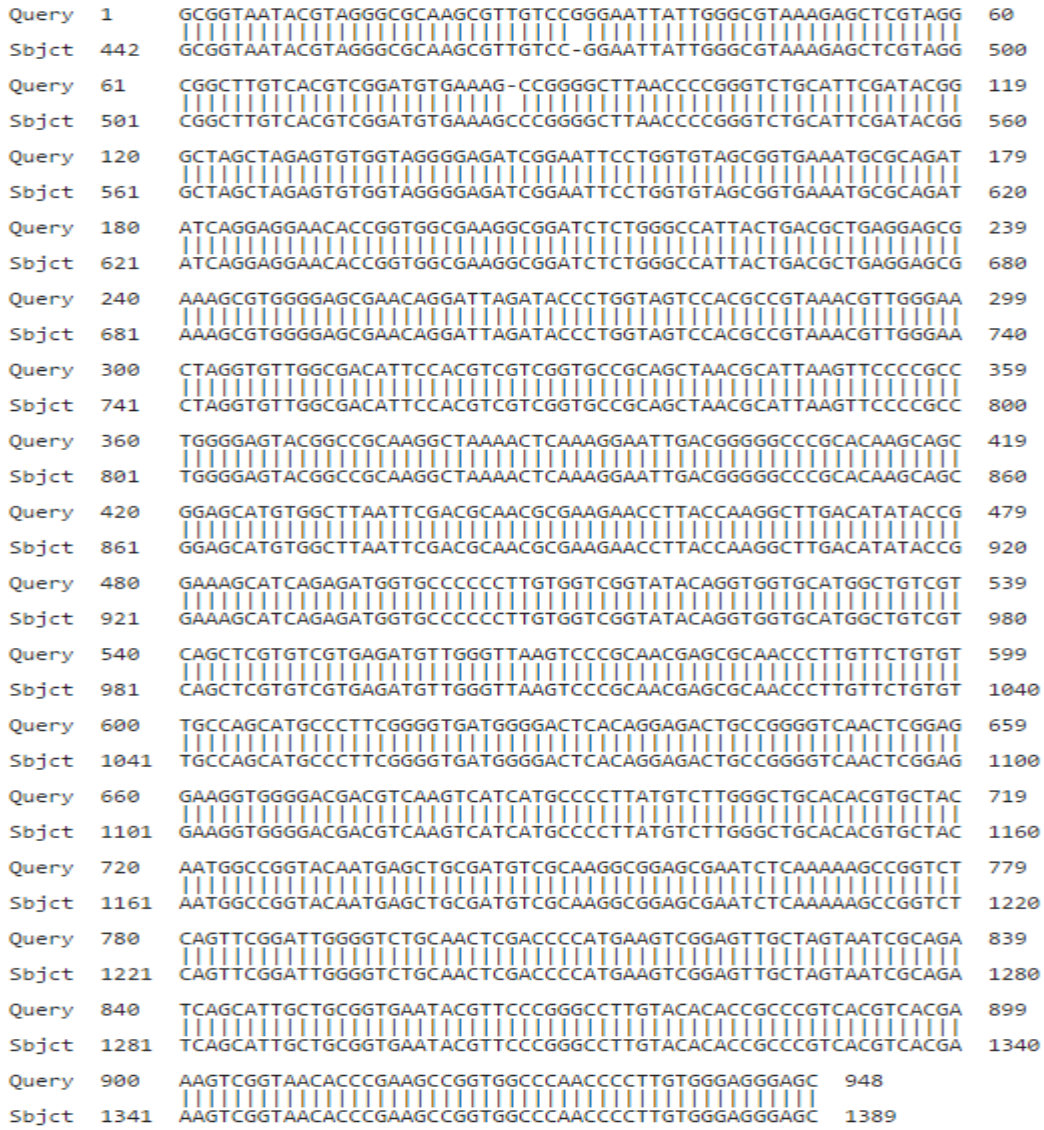
Appendix 1.44: Sequence alignments for La1a isolate with *Penicillium* sp. (ITS)

```
                    10         20         30         40         50
EMBOSS       CCGMSTGAGGGCCCTCTGGGTCCAACCTCCCACCCGTGTTTATTTTACCTTGTTG
             ::::.:::::::::::::::::::::::::::::::::::::::::::::::::
EM_FUN CATTACCGAGTGAGGGCCCTCTGGGTCCAACCTCCCACCCGTGTTTATTTTACCTTGTTG
               10         20         30         40         50         60

          60         70         80         90        100        110
EMBOSS CTTCGGCGGGCCCGCCTTAACTGGCCGCCGGGGGGCTTACGCCCCCGGGCCCGCGCCCGC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_FUN CTTCGGCGGGCCCGCCTTAACTGGCCGCCGGGGGGCTTACGCCCCCGGGCCCGCGCCCGC
               70         80         90        100        110        120

        120        130        140        150        160        170
EMBOSS CGAAGACACCCTCGAACTCTGTCTGAAGATTGTAGTCTGAGTGAAAATATAAATTATTTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_FUN CGAAGACACCCTCGAACTCTGTCTGAAGATTGTAGTCTGAGTGAAAATATAAATTATTTA
               130        140        150        160        170        180

        180        190        200        210        220        230
EMBOSS AAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGAT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_FUN AAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGAT
               190        200        210        220        230        240

        240        250        260        270        280        290
EMBOSS ACGTAATGTGAATTGCAAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_FUN ACGTAATGTGAATTGCAAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCC
               250        260        270        280        290        300

        300        310        320        330        340        350
EMBOSS TGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTTCTGCCCTCAAGCACGGCTTGTGT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_FUN TGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTTCTGCCCTCAAGCACGGCTTGTGT
               310        320        330        340        350        360

        360        370        380        390        400        410
EMBOSS GTTGGGCCCCGTCCTCCGATCCCGGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_FUN GTTGGGCCCCGTCCTCCGATCCCGGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTC
               370        380        390        400        410        420

        420        430        440        450        460        470
EMBOSS CGGTCCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGCCGGCGCTTGCCGA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_FUN CGGTCCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGCCGGCGCTTGCCGA
               430        440        450        460        470        480
        480        490        500
EMBOSS TCAACCCAAATTTTTATCCAGGTTGACCTCGG
       ::::::::::::::::::::::::::::::::
EM_FUN TCAACCCAAATTTTTATCCAGGTTGACCTCGGATCAGGTAG
               490        500        510        520
```

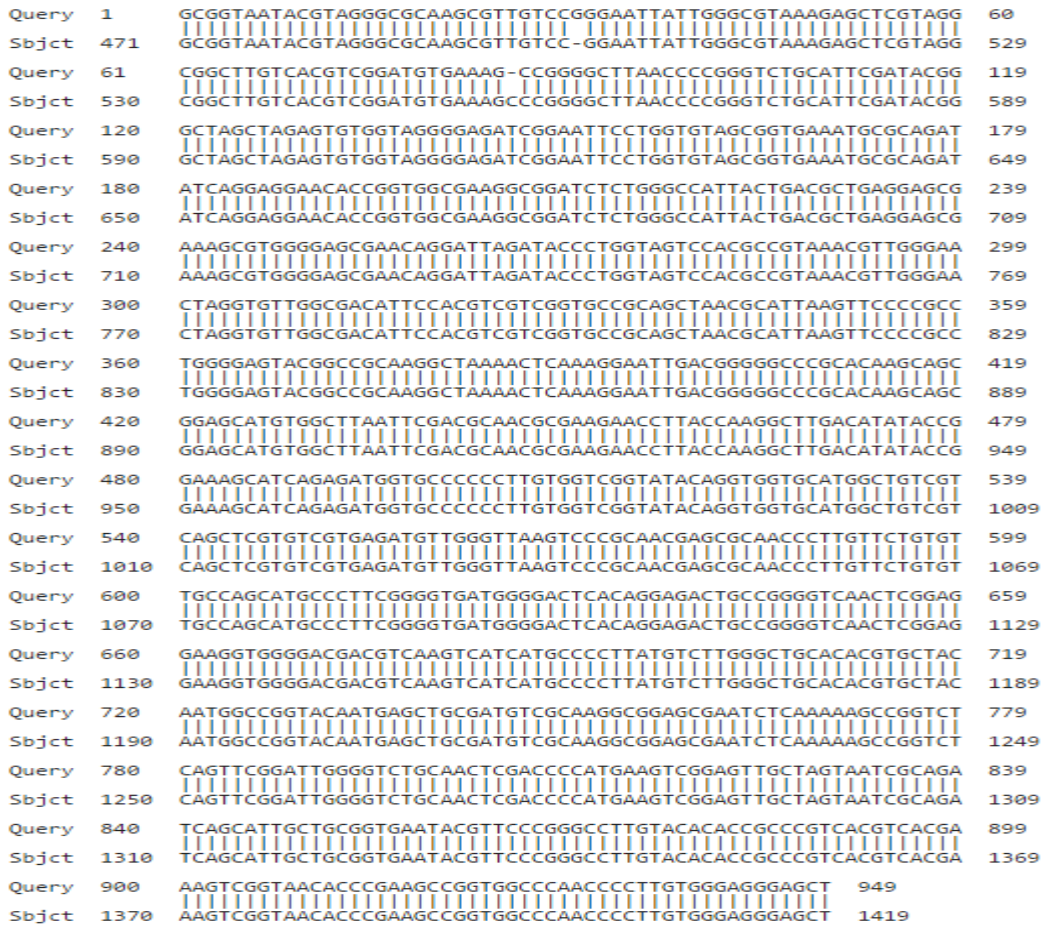
Appendix 1.45: Sequence alignments 0f Es1, M1 11 and La1a (ITS)

```
Laia      CCGMSTGAGGGCCCTCTGGGTCCAACCTCCCACCCGTGTTTATTTTACCTTGTTGCTTCG
ES1       ------GAGGGCCCTCTGGGTCCAACCTCCCACCCGTGTTTATTTTACCTTGTTGCTTCG
M111      ------------CTCTGGGTCCAACCTCCCACCCGTGTTTATTTTACCTTGTTGCTTCG
                      *********************************************

Laia      GCGGGCCCGCCTTAACTGGCCGCCGGGGGGCTTACGCCCCCGGGCCCGCGCCCGCCGAAG
ES1       GCGGGCCCGCCTTAACTGGCCGCCGGGGGGCTTACGCCCCCGGGCCCGCGCCCGCCGAAG
M111      GCGGGCCCGCCTTAACTGGCCGCCGGGGGGCTTACGCCCCCGGGCCCGCGCCCGCCGAAG
          ************************************************************

Laia      ACACCCTCGAACTCTGTCTGAAGATTGTAGTCTGAGTGAAAATATAAATTATTTAAAACT
ES1       ACACCCTCGAACTCTGTCTGAAGATTGTAGTCTGAGTGAAAATATAAATTATTTAAAACT
M111      ACACCCTCGAACTCTGTCTGAAGATTGTAGTCTGAGTGAAAATATAAATTATTTAAAACT
          ************************************************************

Laia      TTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATACGTA
ES1       TTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATACGTA
M111      TTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATACGTA
          ************************************************************

Laia      ATGTGAATTGCAAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTA
ES1       ATGTGAATTGCAAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTA
M111      ATGTGAATTGCAAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTA
          ************************************************************

Laia      TTCCGGGGGGGCATGCCTGTCCGAGCGTCATTTCTGCCCTCAAGCACGGCTTGTGTGTTGG
ES1       TTCCGGGGGGGCATGCCTGTCCGAGCGTCATTTCTGCCCTCAAGCACGGCTTGTGTGTTGG
M111      TTCCGGGGGGGCATGCCTGTCCGAGCGTCATTTCTGCCCTCAAGCACGGCTTGTGTGTTGG
          ************************************************************

Laia      GCCCCGTCCTCCGATCCCGGGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTC
ES1       GCCCCGTCCTCCGATCCCGGGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTC
M111      GCCCCGTCCTCCGATCCCGGGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTC
          ************************************************************

Laia      CTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGCCGGCGCTTGCCGATCAAC
ES1       CTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGCCGGCGCTTGCCGATCAAC
M111      CTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGCCGGCGCTTGCCGATCAAC
          ************************************************************

Laia      CCAAATTTTTATCCAGGTTGACCTCGG----------------------
ES1       CCAAATTTTTATCCAGGTTGACCTCGGATCAGGTAGGGATACCSG-----
M111      CCAAATTTTTATCCAGGTTGACCTCGGATCAGGTAGGGATACCCGCKAAC
          **************************
```

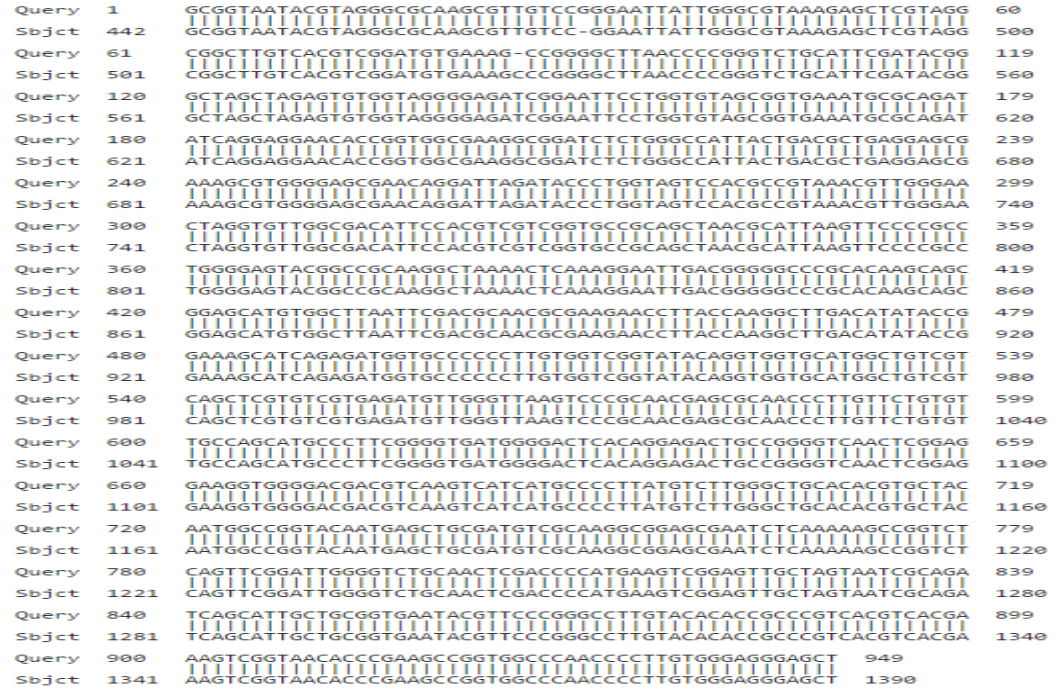
Appendix 1.46: Sequence alignments for Wata 2 isolate with the three closest identification strains



Streptomyces sp SIA-Cu-E1



Streptomyces halstedii strain NRRL B-1238



Streptomyces cyaneus strain ISP 5108

Appendix 1.47: Sequence alignments for Es5 isolate with the three closest identification strains

```
Query  1    CATGCAGTCGAACGATGAAGCCGCTTCGGTGGTGGATTAGTGGCGAACGGGTGAGTAACA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  10   CATGCAGTCGAACGATGAAGCCGCTTCGGTGGTGGATTAGTGGCGAACGGGTGAGTAACA  69

Query  61   CGTGGGCAATCTGCCCTTCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  70   CGTGGGCAATCTGCCCTTCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATA  129

Query  121  ACACTCTGTCCCGCATGGGACGGGGTTAAAATCTCCAGCCCGATAAAGAACTGA-CCC-C  178
            |||||||||||||||||||||||||||||| |||| ||  | | ||| | ||| ||| |
Sbjct  130  ACACTCTGTCCCGCATGGGACGGGGTTAAAAGCTCCGGCG-G-TGAAGGA-TGAGCCCGC  186

Query  179  GGCCTTTTGAGATTGT-GGTCGGGTTAAGGCCT  210
            ||||| |  || |||| ||| |||| | |||||
Sbjct  187  GGCCTATC-AGCTTGTTGGTGGGGTAATGGCCT  218
```

Streptomyces sp GS01

```
Query  1    CATGCAGTCGAACGATGAAGCCGCTTCGGTGGTGGATTAGTGGCGAACGGGTGAGTAACA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6    CATGCAGTCGAACGATGAAGCCGCTTCGGTGGTGGATTAGTGGCGAACGGGTGAGTAACA  65

Query  61   CGTGGGCAATCTGCCCTTCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  66   CGTGGGCAATCTGCCCTTCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATA  125

Query  121  ACACTCTGTCCCGCATGGGACGGGGTTAAAATCTCCAGCCCGATAAAGAACTGA-CCC-C  178
            |||||||||||||||||||||||||||||| |||| ||  | | ||| | ||| ||| |
Sbjct  126  ACACTCTGTCCCGCATGGGACGGGGTTAAAAGCTCCGGCG-G-TGAAGGA-TGAGCCCGC  182

Query  179  GGCCTTTTGAGATTGT-GGTCGGGTTAAGGCCT  210
            ||||| |  || |||| ||| |||| | |||||
Sbjct  183  GGCCTATC-AGCTTGTTGGTGGGGTAATGGCCT  214
```

Streptomyces fimicarius strain G8A-2

```
Query  1    CATGCAGTCGAACGATGAAGCCGCTTCGGTGGTGGATTAGTGGCGAACGGGTGAGTAACA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  21   CATGCAGTCGAACGATGAAGCCGCTTCGGTGGTGGATTAGTGGCGAACGGGTGAGTAACA  80

Query  61   CGTGGGCAATCTGCCCTTCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  81   CGTGGGCAATCTGCCCTTCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATA  140

Query  121  ACACTCTGTCCCGCATGGGACGGGGTTAAAATCTCCAGCCCGATAAAGAACTGA-CCC-C  178
            |||||||||||||||||||||||||||||| |||| ||  | | ||| | ||| ||| |
Sbjct  141  ACACTCTGTCCCGCATGGGACGGGGTTAAAAGCTCCGGCG-G-TGAAGGA-TGAGCCCGC  197

Query  179  GGCCTTTTGAGATTGT-GGTCGGGTTAAGGCCT  210
            ||||| |  || |||| ||| |||| | |||||
Sbjct  198  GGCCTATC-AGCTTGTTGGTGGGGTAATGGCCT  229
```

Streptomyces fimicarius strain G8A-17

Appendix 1.48: Sequence alignments for Pp1 isolate with the three closest identification strains

```
Query  3     AGCACGTTTCGTAAGATTTTGGTTTAAGTCCAAGCAAGTGAGCAAAACCCATGTCC--TG  60
             ||| ||| |||| |||| |||| ||||||||  ||||  ||||  ||||| |||||  ||
Sbjct  1033  AGCTCGTGTCGTGAGATGTTGGGTTAAGTCC-CGCAA-CGAGCGCAACCCTTGTCCCGTG  1090

Query  61    ATTCCCCAGCAGGCCGTTGTGGTGCTGGGGATTCACGGGAGACCCCCGGGGTCAACTCGG  120
              || ||||||||||| |||||||||||||| |||||||||||| ||||||||||||||||
Sbjct  1091  -TT-CCCAGCAGGCCCTTGTGGTGCTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGG  1148

Query  121   AGGAAGGTGGGGAAGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGGT  180
             |||||||||||| |||||||||||||||||||||||||||||||||||||||||||||| |
Sbjct  1149  AGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCT  1208

Query  181   CCAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGT  240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1209  ACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGT  1268

Query  241   CTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCA  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1269  CTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCA  1328

Query  301   GATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCAC  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1329  GATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCAC  1388

Query  361   GAAAGTCGGTAACACCCGAAGCCGGTGGCCCAACCCCTTGTGGGAGGGA  409
             |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1389  GAAAGTCGGTAACACCCGAAGCCGGTGGCCCAACCCCTTGTGGGAGGGA  1437
```

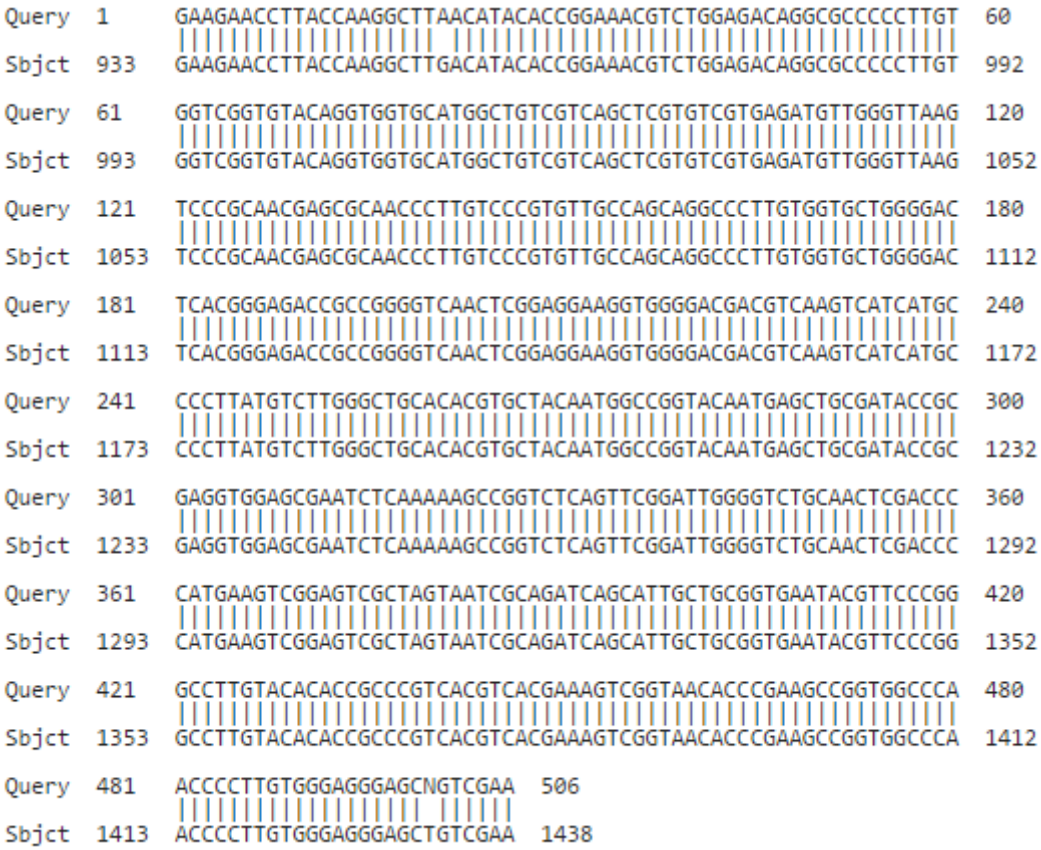
Streptomyces sp GY2

```
Query  3     AGCACGTTTCGTAAGATTTTGGTTTAAGTCCAAGCAAGTGAGCAAAACCCATGTCCTGAT  62
             ||| ||| |||| |||| |||| ||||||||  ||||  ||||  ||||| |||||||
Sbjct  983   AGCTCGTGTCGTGAGATGTTGGGTTAAGTCC-CGCAA-CGAGCGCAACCCTTGTCCTGTG  1040

Query  63    TCCCCAGCAGGCCGTTGTGGTGCTGGGGATTCACGGGAGACCCCCGGGGTCAACTCGGAG  122
             |  |||||||||| |||||||||||||| |||||||||||| ||||||||||||||||||
Sbjct  1041  TTGCCAGCAGGCCCTTGTGGTGCTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAG  1100

Query  123   GAAGGTGGGGAAGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGGTCC  182
             |||||||||||| |||||||||||||||||||||||||||||||||||||||||||| | |
Sbjct  1101  GAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTAC  1160

Query  183   AATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCT  242
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1161  AATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCT  1220

Query  243   CAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGA  302
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1221  CAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGA  1280

Query  303   TCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGA  362
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1281  TCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGA  1340

Query  363   AAGTCGGTAACACCCGAAGCCGGTGGCCCAACCCCTTGTGGGAGGGA  409
             |||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1341  AAGTCGGTAACACCCGAAGCCGGTGGCCCAACCCCTTGTGGGAGGGA  1387
```

Streptomyces sp C3001

Streptomyces sp. strain ARR4

Appendix 1.49: Sequence alignments for Pp 2 isolate with the three closest identification strains



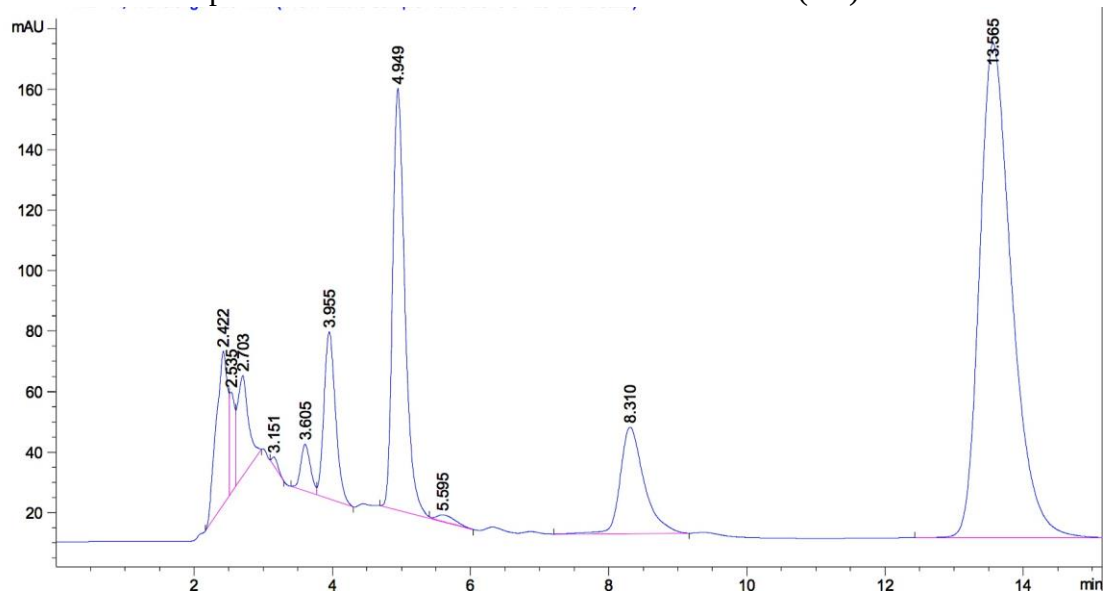
Streptomyces sp. strain 194

Streptomyces violaceus strain RTAE11

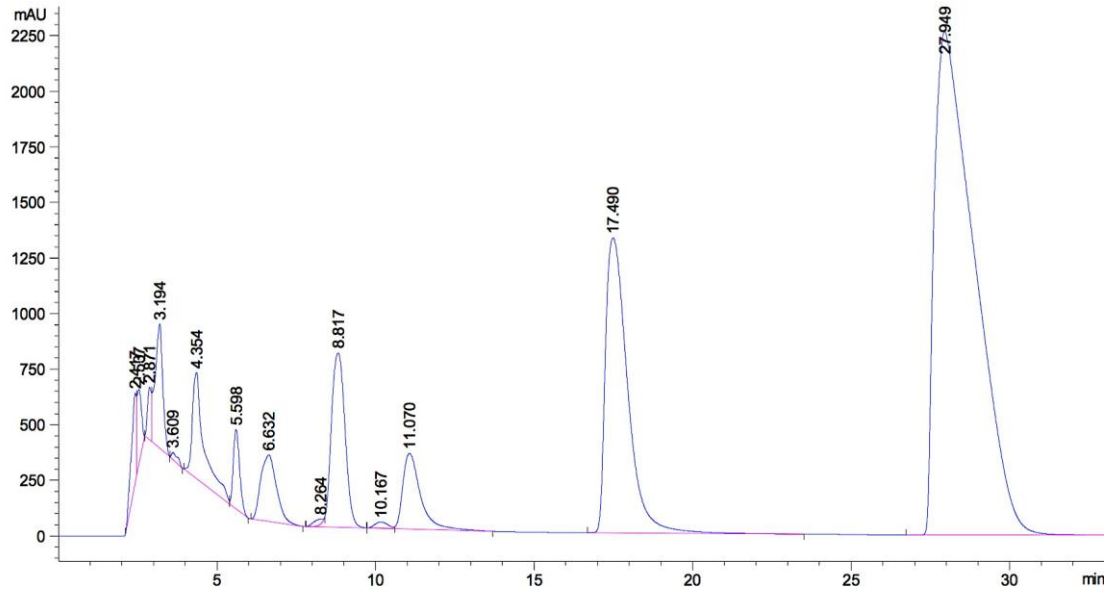
Streptomyces sp. strain CAE3

Section 2: HPLC chromatograms

Appendix 2.1: Fractionation of Es1 extracts by HPLC on Spherisorb using an isocratic mobile phase of 0.1% TFA in water: methanol 30:70 (v/v)

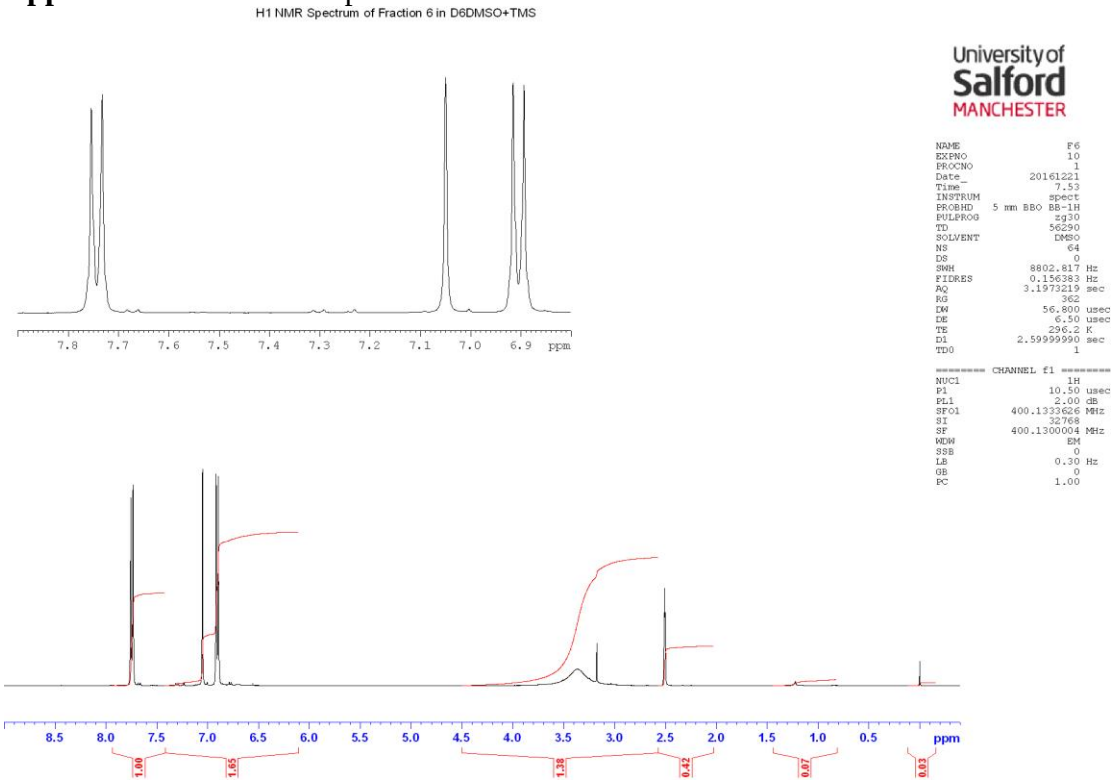


Appendix 2.2: Fractions obtained by injecting 250 µl Es1 extracts on to a semi-Preparative ACE Excel 5 C-18 amide column, flow rate 5 ml/min, the solvent systems was Gradient mobile phase water: methanol 30:70 (v/v).

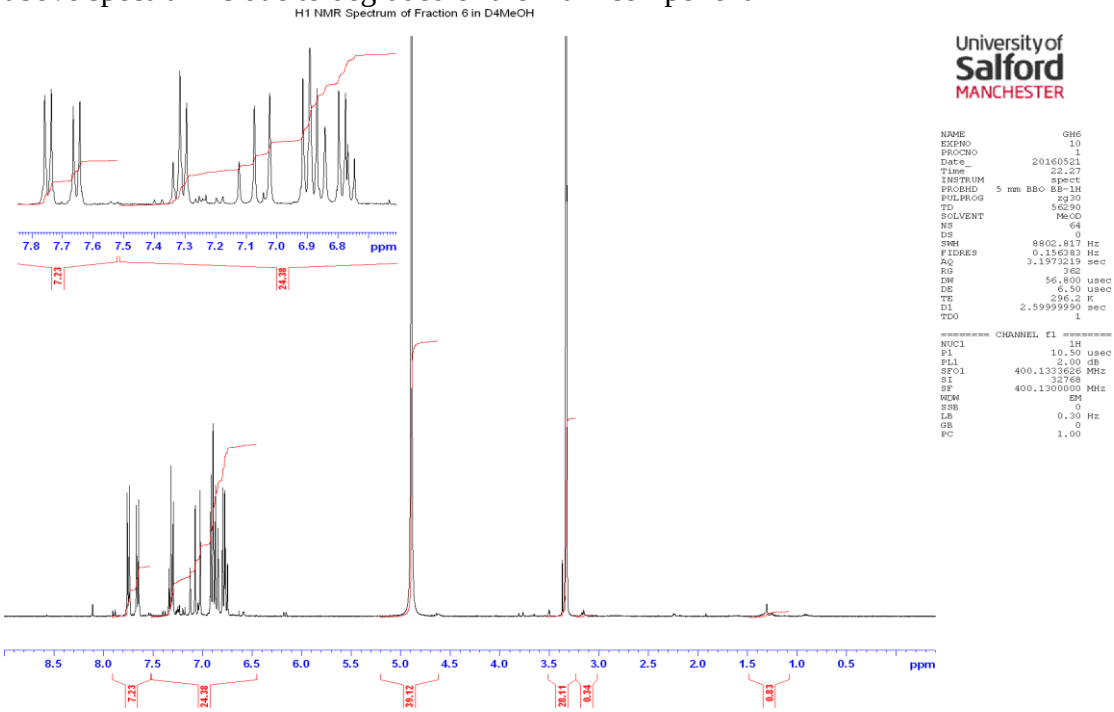


Section 3: NMR, MS and FTIR data

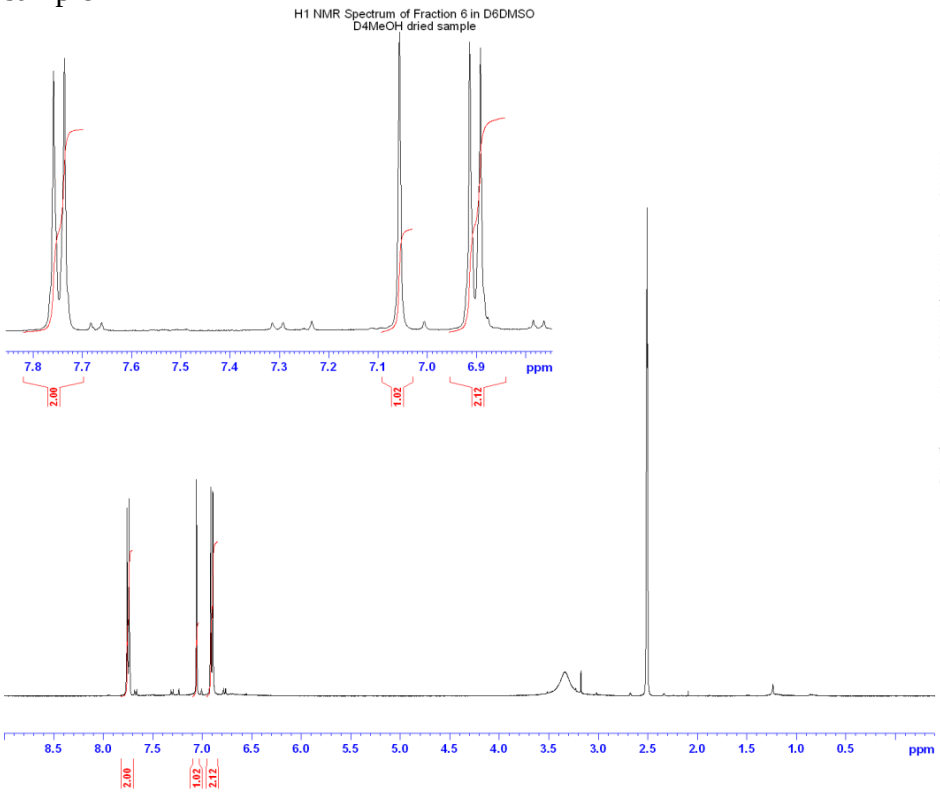
Appendix 3.1: ^{1}H NMR Spectrum of F6 in D6DMSO+TMS



Appendix 3.2: ^{1}H NMR Spectrum of F6 in CD_3OD+TMS, the peaks observed in the above spectrum is due to degraded of the main component



Appendix 3.3: ^{1}H NMR Spectrum of F6 in D6DMSO+TMS after CD_3OD dried sample



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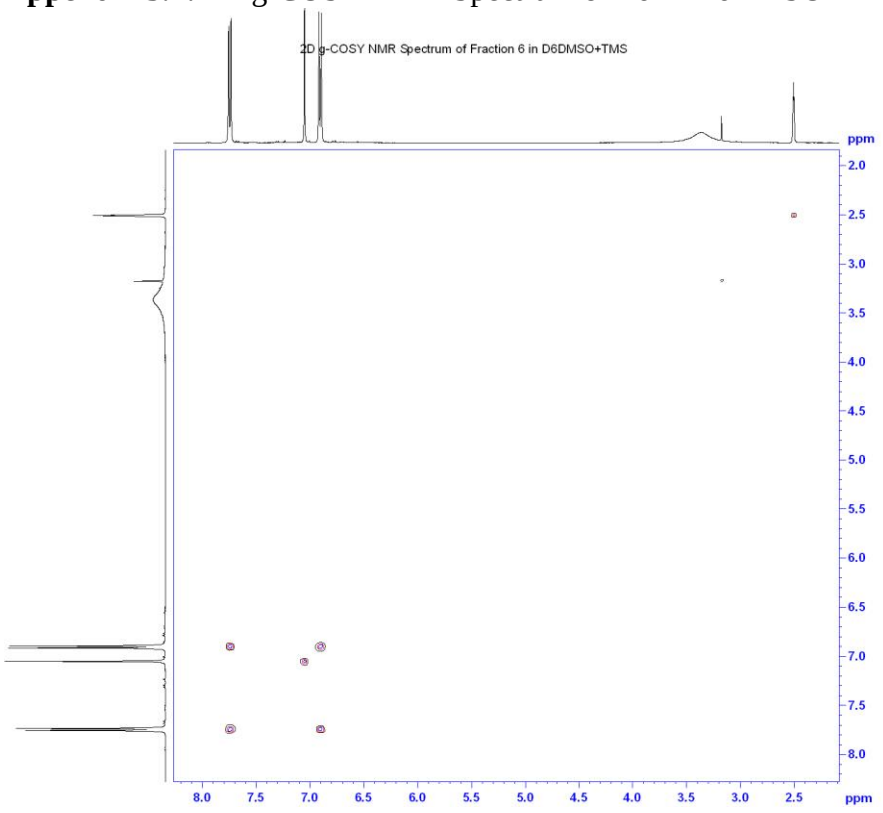
```
NAME                GHFr6
EXPNO                  20
PROCNO                  1
Date_            20160524
Time                14.06
INSTRUM             spect
PROBHD   5 mm BBO BB-1H
PULPROG             zg30
TD                 56290
SOLVENT             DMSO
NS                     64
DS                      0
SWH             8802.817 Hz
FIDRES          0.156383 Hz
AQ              3.1973219 sec
RG                    362
DW                56.800 usec
DE                 6.50 usec
TE                296.2 K
D1             2.59999990 sec
TD0                     1

======== CHANNEL f1 ========
NUC1                   1H
P1                 10.50 usec
PL1                 2.00 dB
SFO1          400.1333626 MHz
SI                  32768
SF            400.1300000 MHz
WDW                    EM
SSB                     0
LB                 0.30 Hz
GB                      0
PC                   1.00
```

Appendix 3.4: 2D g-COSY NMR Spectrum of F6 in D6DMSO+TMS



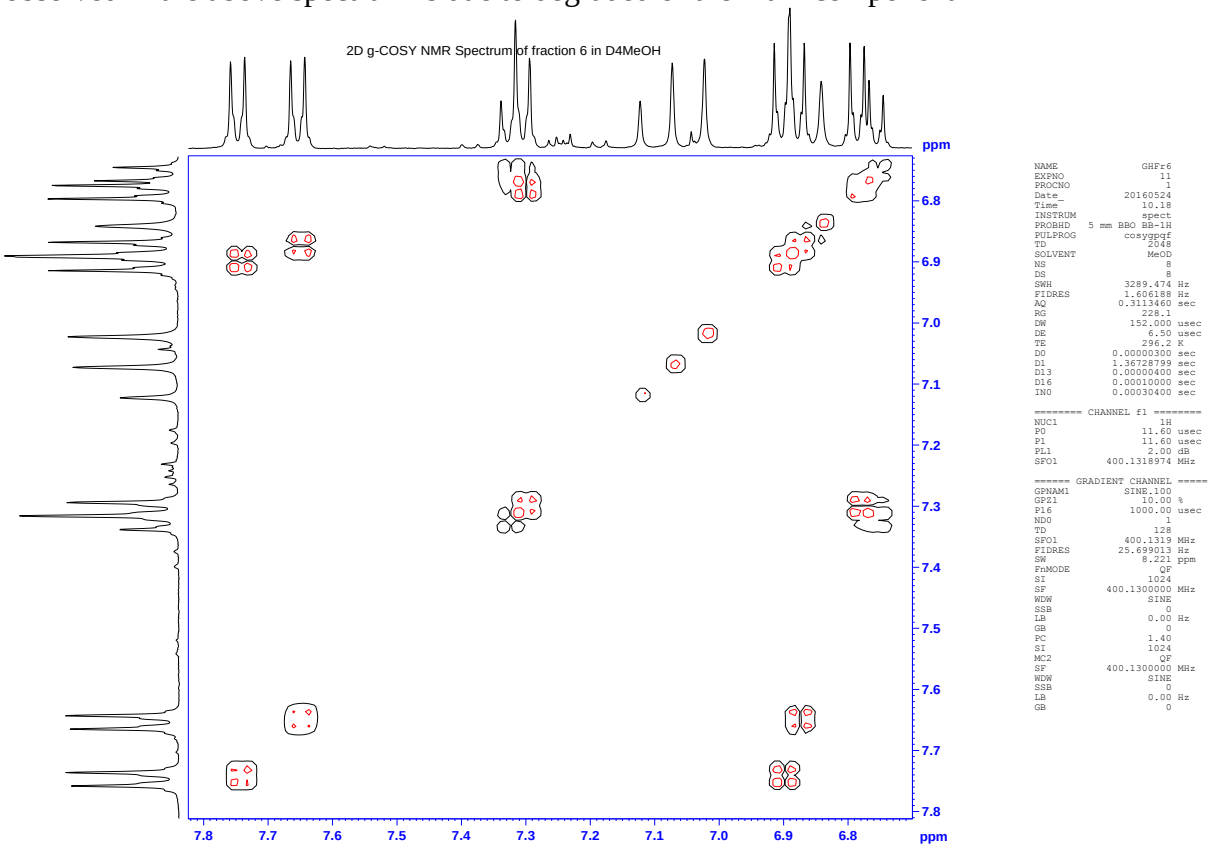
University of Salford MANCHESTER

```
NAME                   F6
EXPNO                  15
PROCNO                  1
Date_            20161221
Time                12.54
INSTRUM             spect
PROBHD   5 mm BBO BB-1H
PULPROG          cosygpqf
TD                  2048
SOLVENT             DMSO
NS                     8
DS                     8
SWH             4595.588 Hz
FIDRES          2.243940 Hz
AQ              0.2228724 sec
RG                   128
DW                108.800 usec
DE                 6.50 usec
TE                296.2 K
D0           0.00000300 sec
D1           1.45535195 sec
D13          0.00000400 sec
D16          0.00010000 sec
IN0          0.00021760 sec

======== CHANNEL f1 ========
NUC1                   1H
P0                 11.60 usec
P1                 11.60 usec
PL1                 2.00 dB
SFO1          400.1324020 MHz

====== GRADIENT CHANNEL =====
GPNAM1          SINE.100
GPZ1               10.00 %
P16              1000.00 usec
ND0                     1
TD                    128
SFO1          400.1324 MHz
FIDRES         35.903034 Hz
SW                 11.485 ppm
FnMODE                 QF
SI                   1024
SF            400.1300004 MHz
WDW                  SINE
SSB                     0
LB                 0.00 Hz
GB                      0
PC                   1.40
SI                   1024
MC2                    QF
SF            400.1300004 MHz
WDW                  SINE
SSB                     0
LB                 0.00 Hz
GB                      0
```

Appendix 3.5: 2D g-COSY NMR Spectrum of F6 (expansion) in CD_3OD, the peaks observed in the above spectrum is due to degraded of the main component

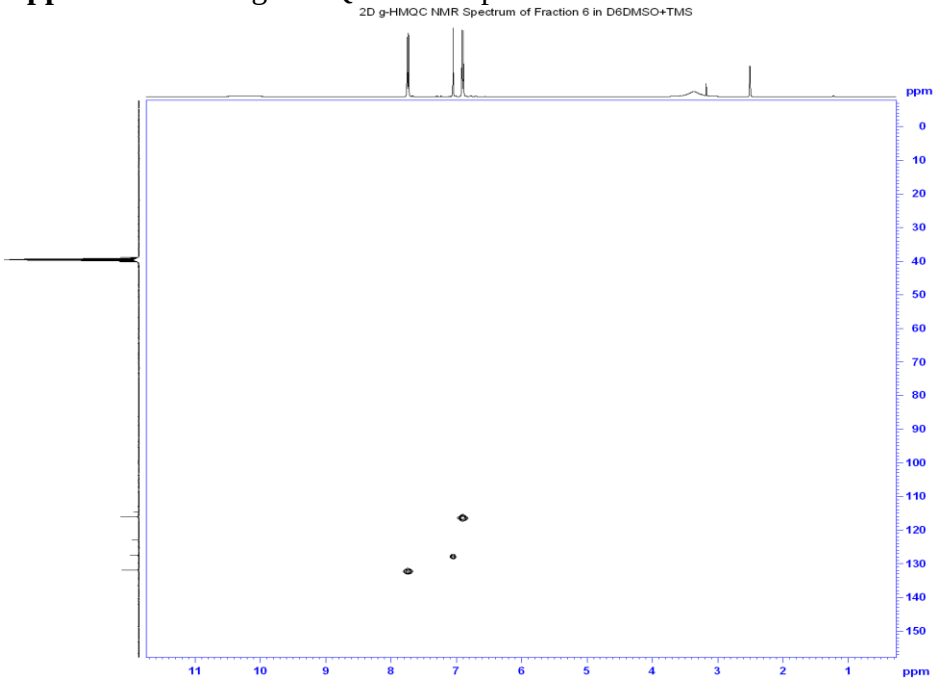



```
NAME                  GHFr6
EXPNO                    11
PROCNO                    1
Date_              20160524
Time                  10.18
INSTRUM                spect
PROBHD     5 mm BBO BB-1H
PULPROG           cosygpqf
TD                    2048
SOLVENT               MeOD
NS                       8
DS                       8
SWH                3289.474 Hz
FIDRES             1.606188 Hz
AQ                 0.3113460 sec
RG                     228.1
DW                   152.000 usec
DE                      6.50 usec
TE                    296.2 K
D0                 0.00000300 sec
D1                 1.36728799 sec
D13                0.00000400 sec
D16                0.00010000 sec
IN0                0.00030400 sec

======== CHANNEL f1 ========
NUC1                      1H
P0                     11.60 usec
P1                     11.60 usec
PL1                     2.00 dB
SFO1           400.1318974 MHz

====== GRADIENT CHANNEL =====
GPNAM1              SINE.100
GPZ1                   10.00 %
P16                  1000.00 usec
ND0                       1
TD                      128
SFO1                400.1319 MHz
FIDRES             25.699013 Hz
SW                     8.221 ppm
FnMODE                    QF
SI                      1024
SF              400.1300000 MHz
WDW                     SINE
SSB                        0
LB                      0.00 Hz
GB                         0
PC                      1.40
SI                      1024
MC2                       QF
SF              400.1300000 MHz
WDW                     SINE
SSB                        0
LB                      0.00 Hz
GB                         0
```

Appendix 3.6: 2D g-HMQC NMR Spectrum of F6 in D6DMSO+TMS



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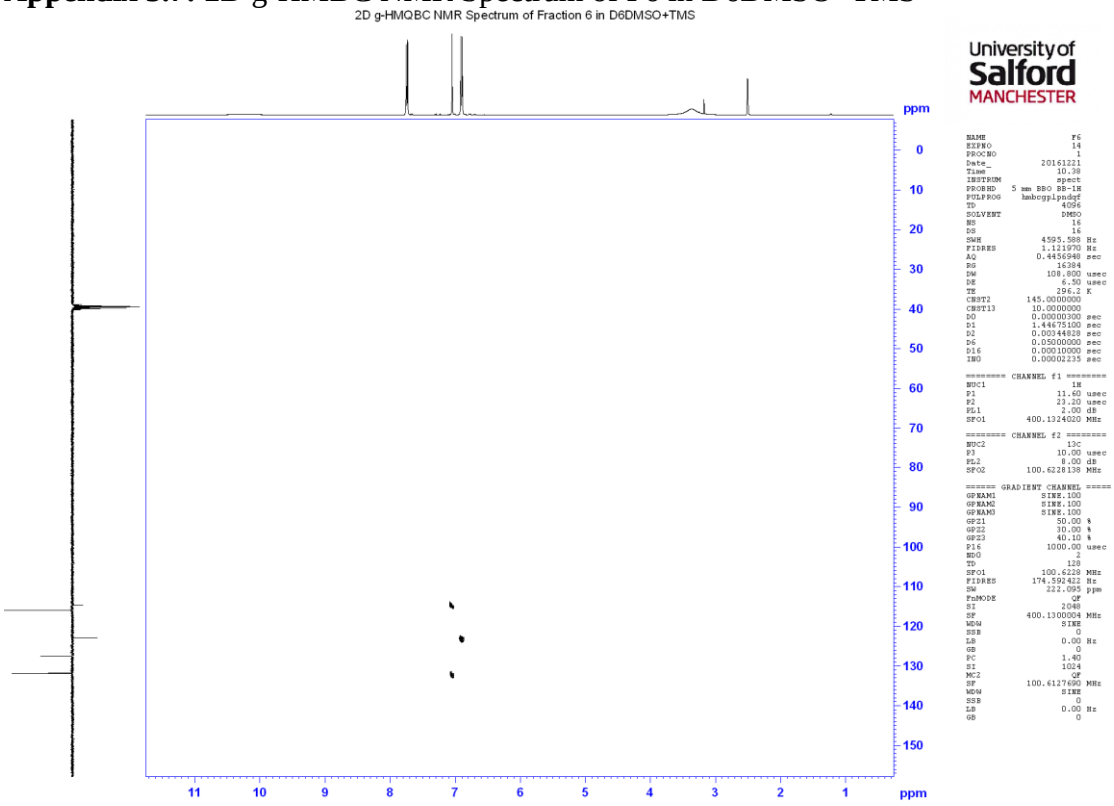
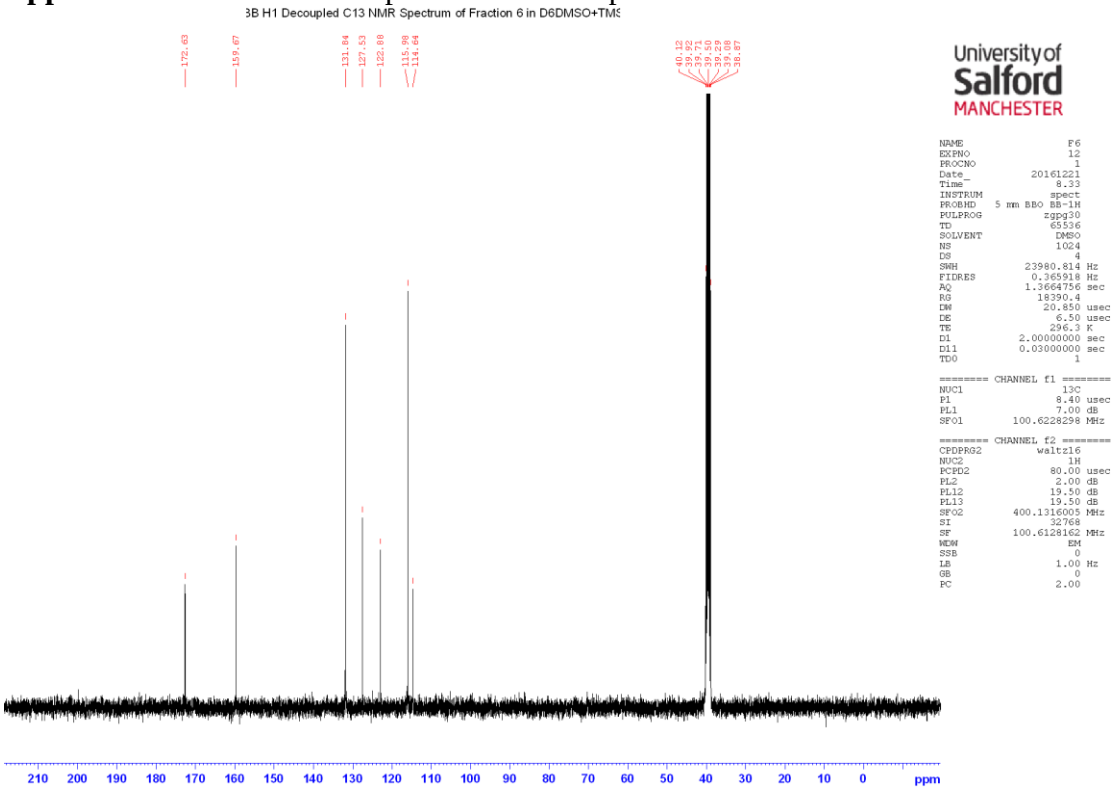
```
NAME                     F6
EXPNO                    11
PROCNO                    1
Date_              20161221
Time                   9.34
INSTRUM                spect
PROBHD     5 mm BBO BB-1H
PULPROG          hmqcgpqf
TD                    1024
SOLVENT               DMSO
NS                       4
DS                      16
SWH                4595.588 Hz
FIDRES             4.489879 Hz
AQ                 0.1114612 sec
RG                    4507.4
DW                   108.800 usec
DE                      6.50 usec
TE                    296.2 K
CNST2              145.0000000
D0                 0.00000100 sec
D1                 1.51516000 sec
D2                 0.00344828 sec
D12                0.00002000 sec
D13                0.00000400 sec
D16                0.00010000 sec
IN0                0.00003000 sec

======== CHANNEL f1 ========
NUC1                      1H
P1                     11.60 usec
P2                     23.20 usec
PL1                     2.00 dB
SFO1           400.1324020 MHz

======== CHANNEL f2 ========
CPDPRG2                 waltz
NUC2                      13C
P3                     10.00 usec
PCPD2                  80.00 usec
PL2                     8.00 dB
PL12                   26.04 dB
SFO2           100.6203140 MHz

====== GRADIENT CHANNEL =====
GPNAM1              SINE.100
GPNAM2              SINE.100
GPNAM3              SINE.100
GPZ1                   50.00 %
GPZ2                   30.00 %
GPZ3                   40.10 %
P16                  1000.00 usec
ND0                       2
TD                      128
SFO1                100.6203 MHz
FIDRES             130.208328 Hz
SW                   165.639 ppm
FnMODE                    QF
SI                      1024
SF              400.1300004 MHz
WDW                    QSINE
SSB                        2
LB                      0.00 Hz
GB                         0
PC                      1.40
SI                      1024
MC2                       QF
SF              100.6127690 MHz
WDW                    QSINE
SSB                        2
LB                      0.00 Hz
GB                         0
```

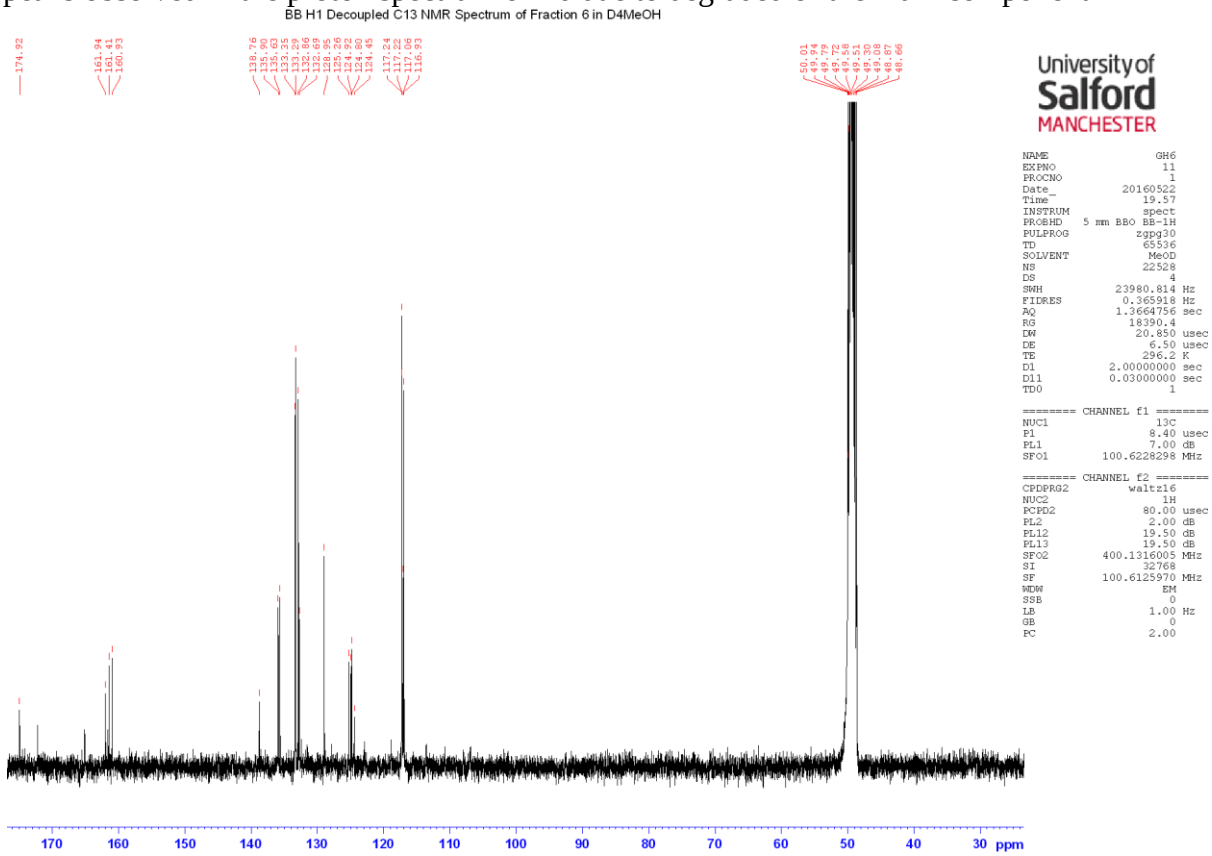
Appendix 3.7: 2D g-HMBC NMR Spectrum of F6 in D6DMSO+TMS



Appendix 3.8: BB H1 Decoupled C13 NMR Spectrum of F6 in D6DMSO+TMS



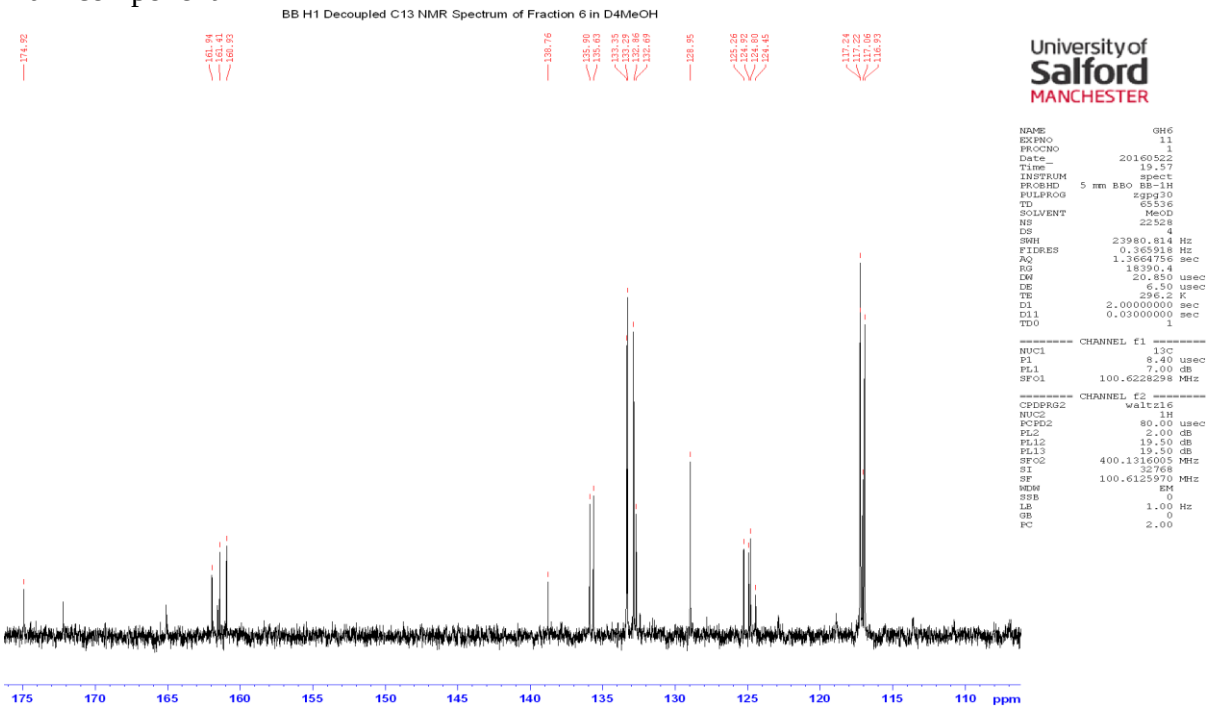
Appendix 3.9: BB H1 Decoupled C13 NMR Spectrum of F6 in CD_3OD, after the peaks observed in the proton spectrum of F6 due to degraded of the main component



University of Salford MANCHESTER

NAME	GH6
EXPNO	11
PROCNO	1
Date_	20160522
Time	19.57
INSTRUM	spect
PROBHD	5 mm BBO BB-1H
PULPROG	zgpg30
TD	65536
SOLVENT	MeOD
NS	22528
DS	4
SWH	23980.814 Hz
FIDRES	0.365918 Hz
AQ	1.3664756 sec
RG	18390.4
DW	20.850 usec
DE	6.50 usec
TE	296.2 K
D1	2.00000000 sec
D11	0.03000000 sec
TD0	1
======== CHANNEL f1 ========	
NUC1	13C
P1	8.40 usec
PL1	7.00 dB
SFO1	100.6228298 MHz
======== CHANNEL f2 ========	
CPDPRG2	waltz16
NUC2	1H
PCPD2	80.00 usec
PL2	2.00 dB
PL12	19.50 dB
PL13	19.50 dB
SFO2	400.1316005 MHz
SI	32768
SF	100.6125970 MHz
WDW	EM
SSB	0
LB	1.00 Hz
GB	0
PC	2.00

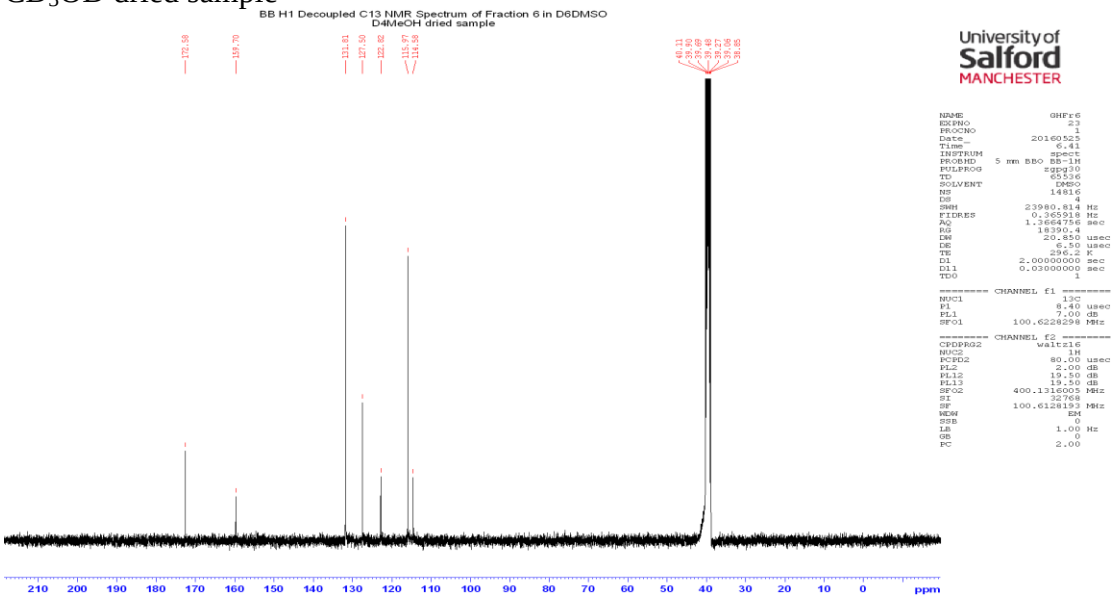
Appendix 3.10: BB H1 Decoupled C13 NMR Spectrum of F6 (expansion) in CD_3OD, after the peaks observed in the proton spectrum of F6 due to degraded of the main component



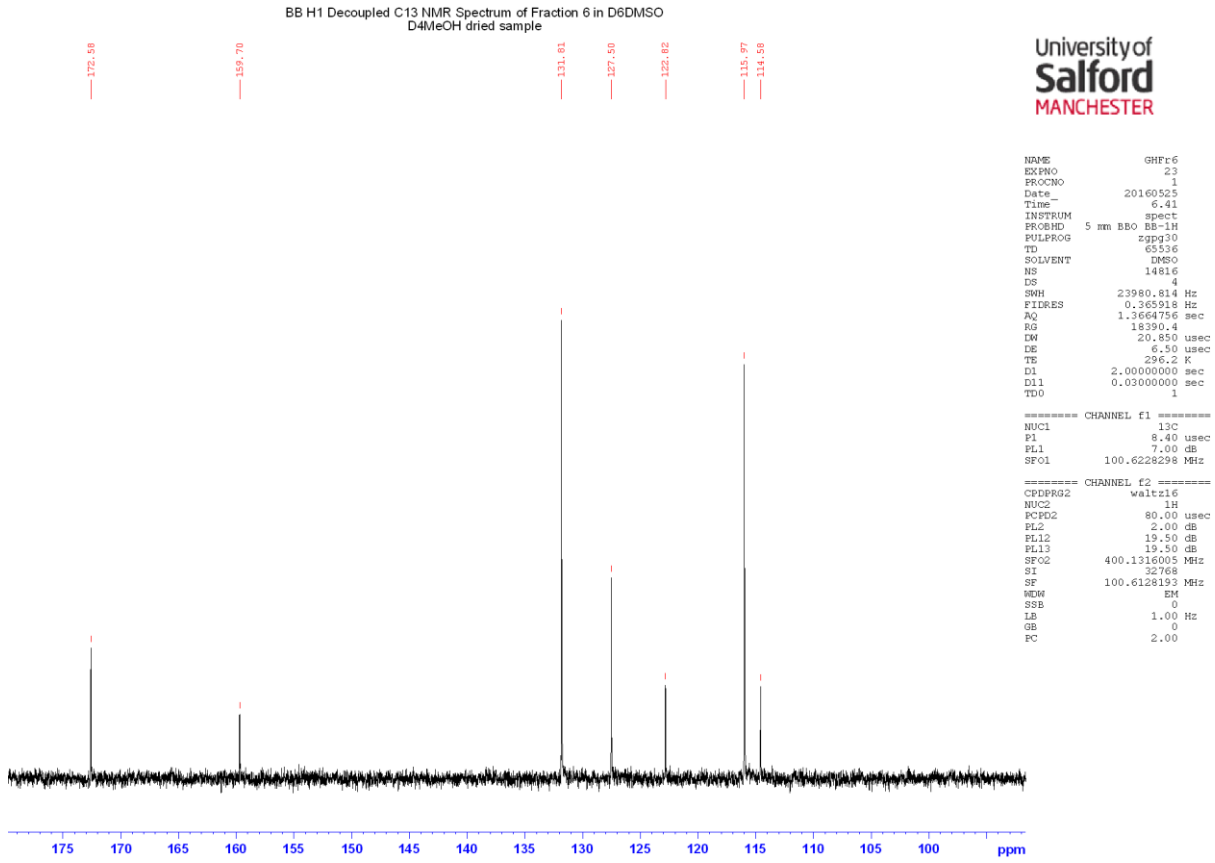
University of Salford MANCHESTER

NAME	GH6
EXPNO	11
PROCNO	1
Date_	20160522
Time	19.57
INSTRUM	spect
PROBHD	5 mm BBO BB-1H
PULPROG	zgpg30
TD	65536
SOLVENT	MeOD
NS	22528
DS	4
SWH	23980.814 Hz
FIDRES	0.365918 Hz
AQ	1.3664756 sec
RG	18390.4
DW	20.850 usec
DE	6.50 usec
TE	296.2 K
D1	2.00000000 sec
D11	0.03000000 sec
TD0	1
======== CHANNEL f1 ========	
NUC1	13C
P1	8.40 usec
PL1	7.00 dB
SFO1	100.6228298 MHz
======== CHANNEL f2 ========	
CPDPRG2	waltz16
NUC2	1H
PCPD2	80.00 usec
PL2	2.00 dB
PL12	19.50 dB
PL13	19.50 dB
SFO2	400.1316005 MHz
SI	32768
SF	100.6125970 MHz
WDW	EM
SSB	0
LB	1.00 Hz
GB	0
PC	2.00

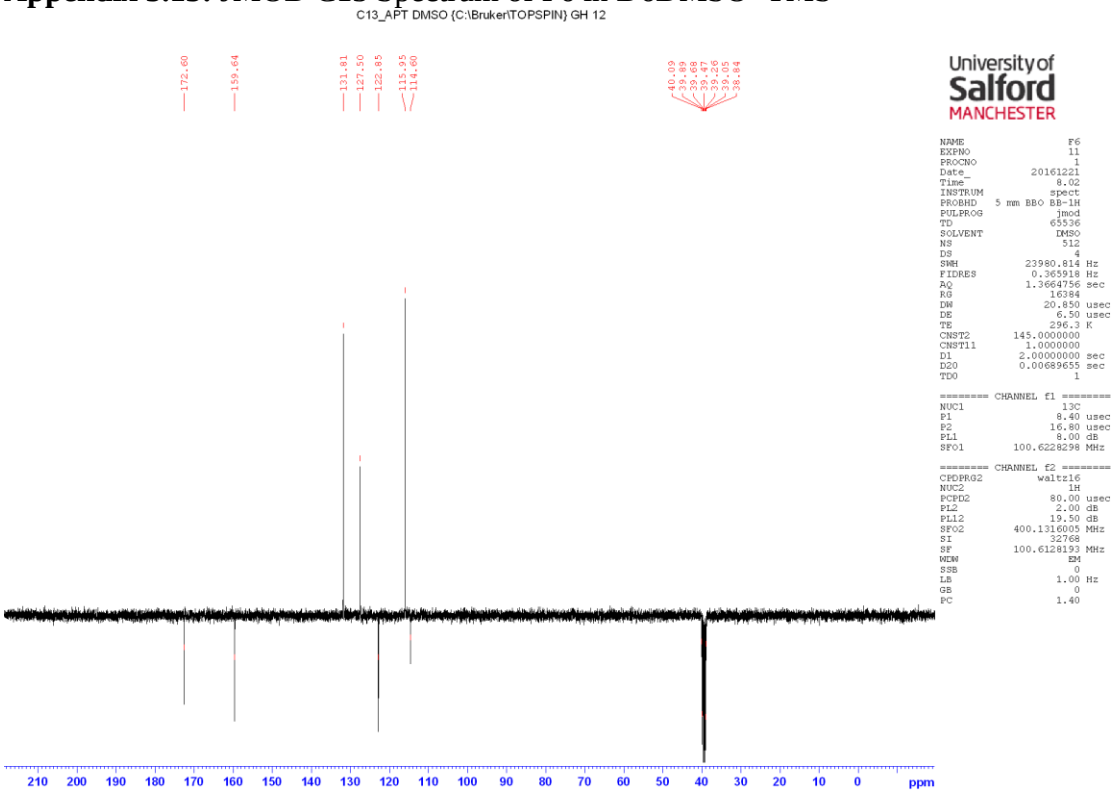
Appendix 3.11: BB H1 Decoupled C13 NMR Spectrum of F6 in D6DMSO after CD_3OD dried sample



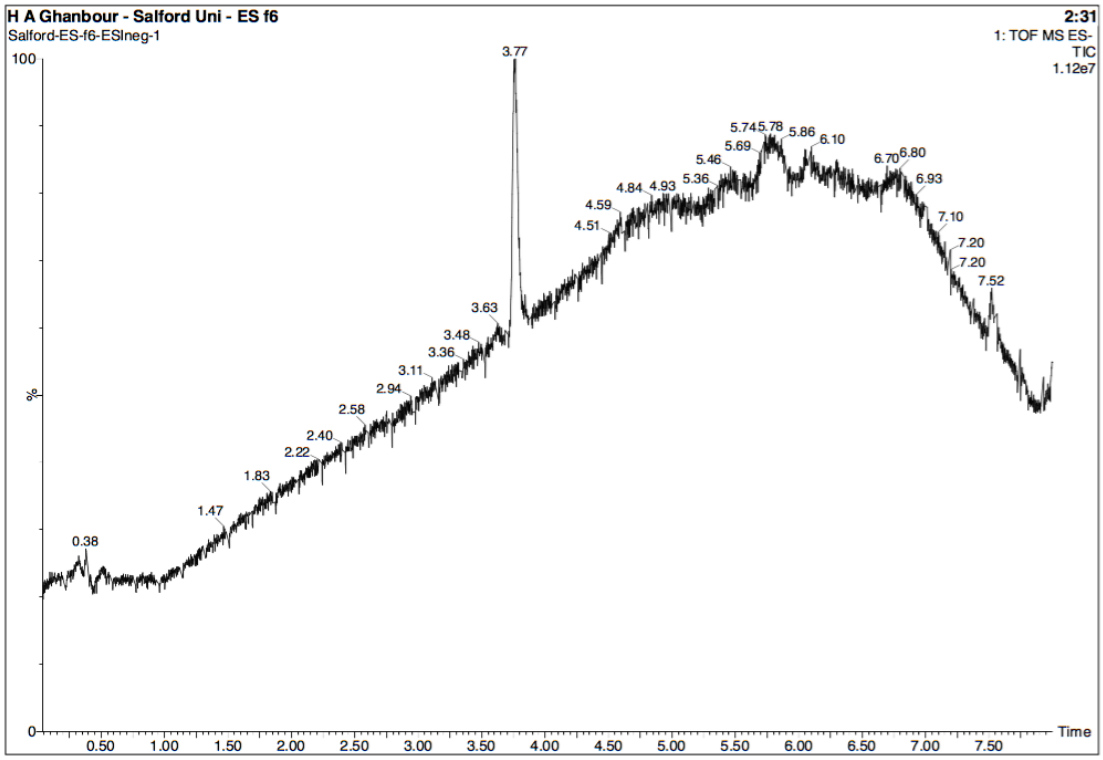
Appendix 3.12: BB H1 Decoupled C13 NMR Spectrum of F6 (expansion) in D6DMSO after CD_3OD dried sample



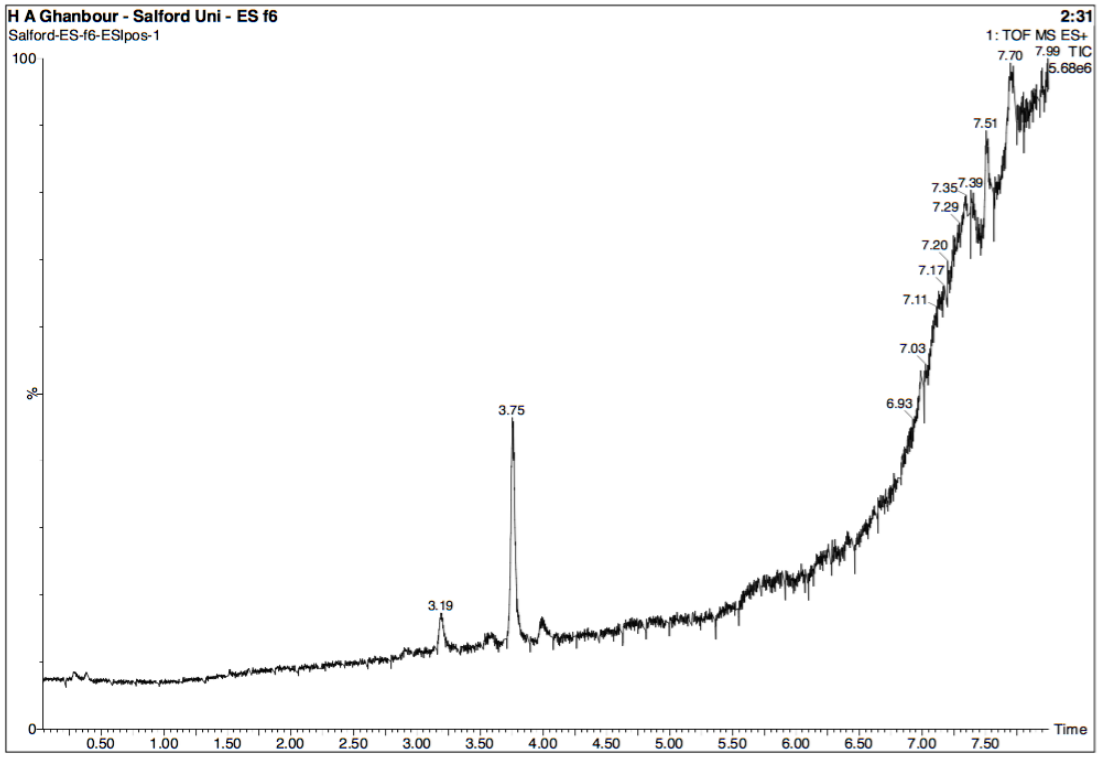
Appendix 3.13: JMOD C13 Spectrum of F6 in D6DMSO+TMS



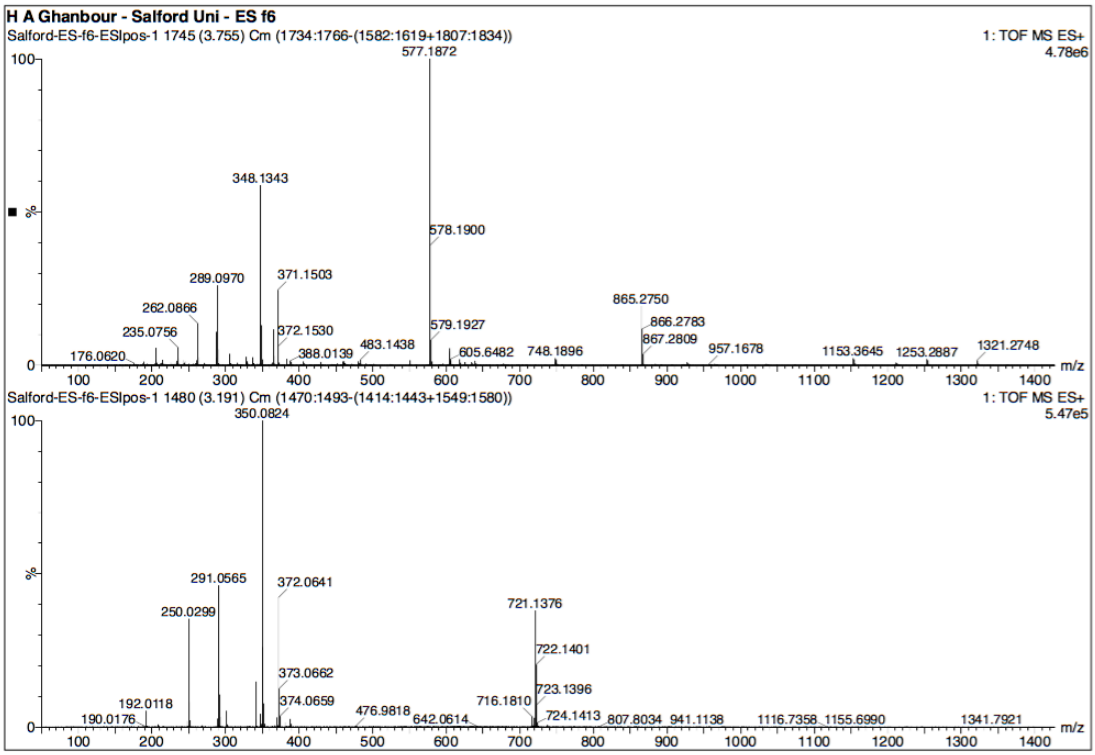
Appendix 3.14: The negative ion chromatogram from LC MS-TOF of F6



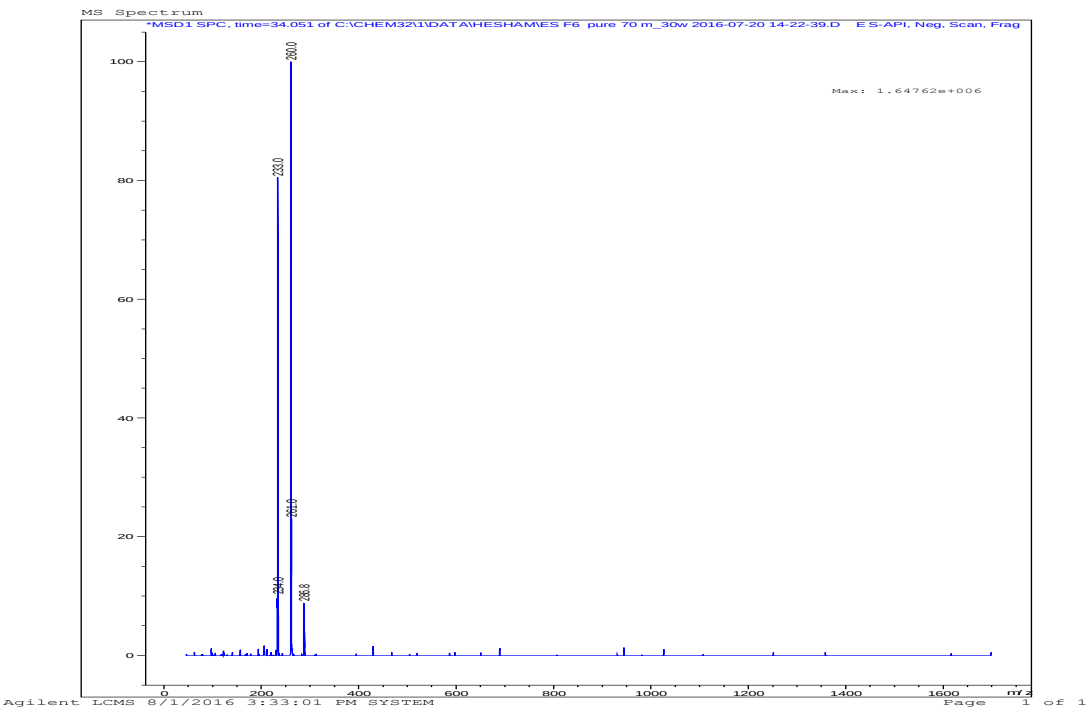
Appendix 3.15: The positive ion chromatogram from LC MS-TOF of F6



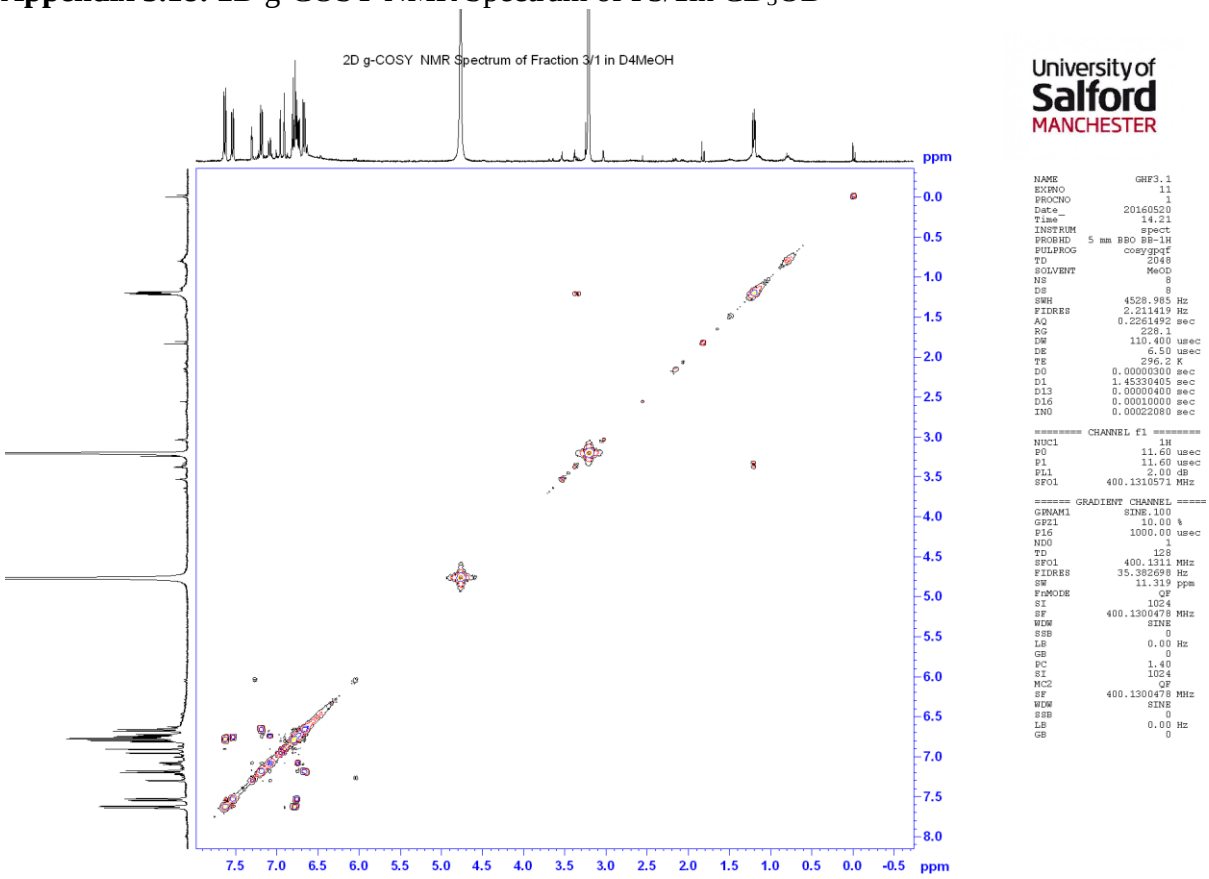
Appendix 3.16: The positive ion spectrum from LC MS-TOF of F6



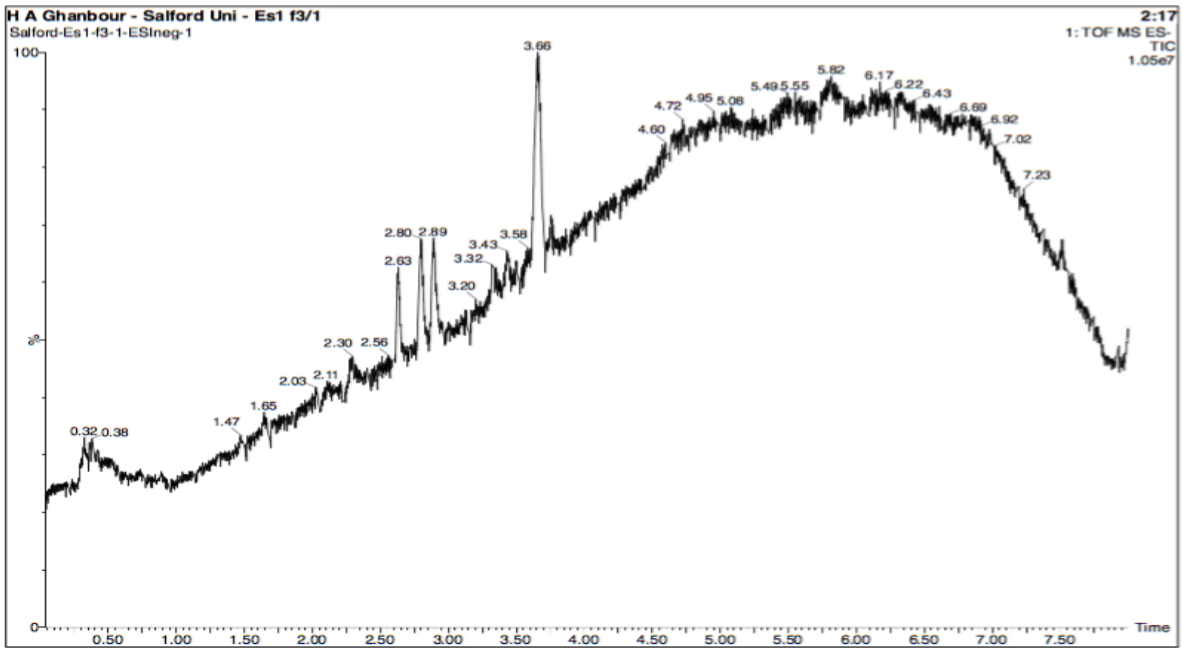
Appendix 3.17: The negative ion spectrum from LC MS (Salford University) of F6



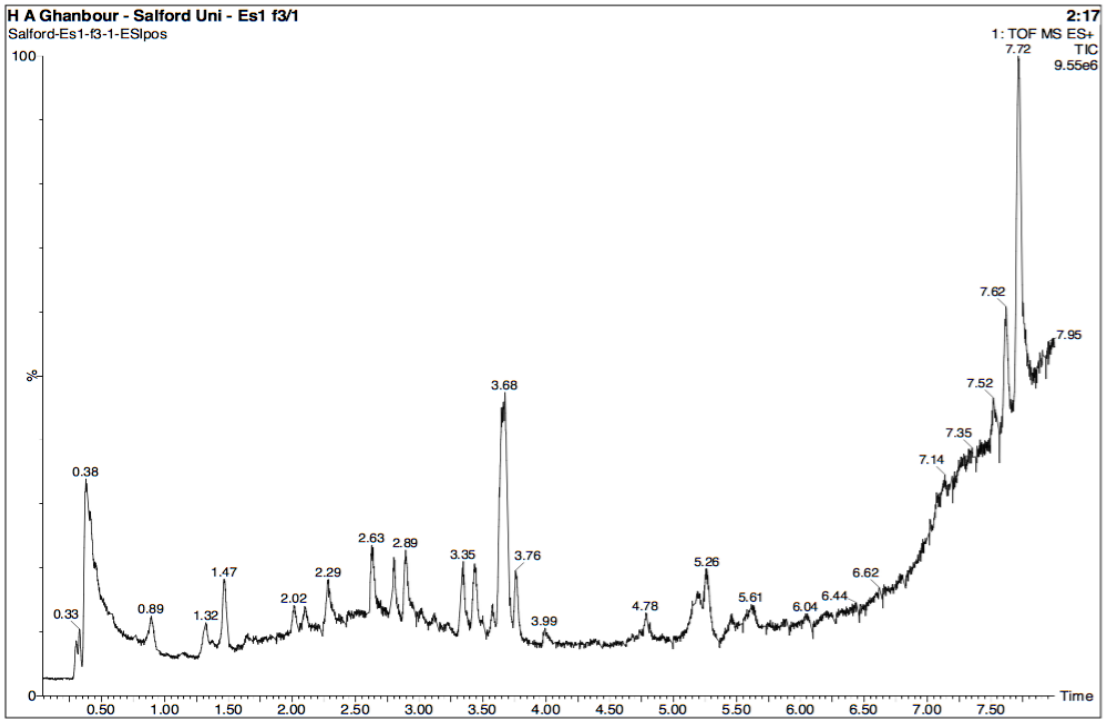
Appendix 3.18: 2D g-COSY NMR Spectrum of F3/1in CD_3OD



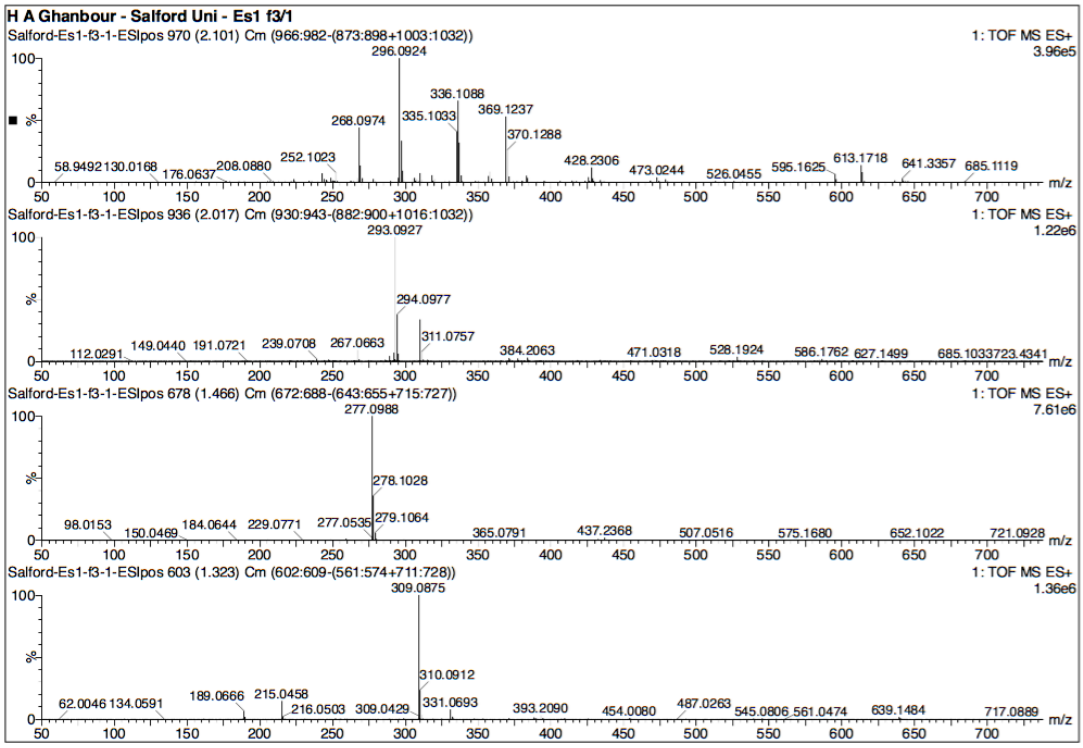
Appendix 3.19: The negative ion chromatogram from LC MS-TOF of F3/1



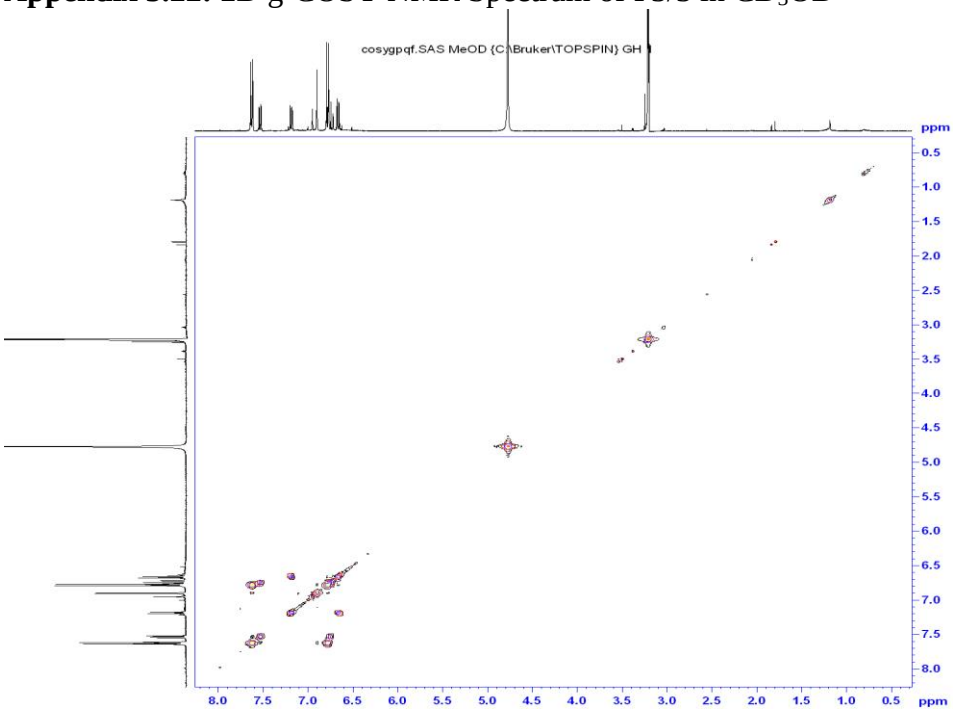
Appendix 3.20: The positive ion chromatogram from LC MS-TOF of F3/1



Appendix 3.21: The positive ion spectrum from LC MS-TOF of F3/1



Appendix 3.22: 2D g-COSY NMR Spectrum of F3/3 in CD_3OD



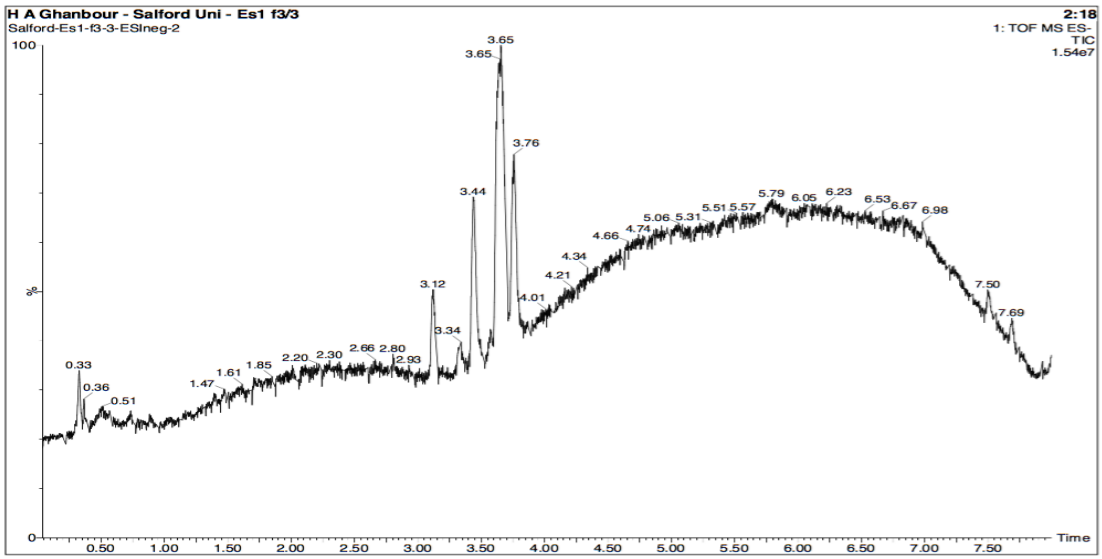
University of Salford MANCHESTER

NAME	GHF3.3
EXPNO	15
PROCNO	1
Date_	20160524
Time	8.45
INSTRUM	spect
PROBHD	5 mm BBO BB-1H
PULPROG	cosygpqf
TD	2048
SOLVENT	MeOD
NS	8
DS	8
SWH	3205.128 Hz
FIDRES	1.565004 Hz
AQ	0.3195380 sec
RG	181
DW	156.000 usec
DE	6.50 usec
TE	296.2 K
D0	0.00000300 sec
D1	1.35909605 sec
D13	0.00000400 sec
D16	0.00010000 sec
IN0	0.00031200 sec
======== CHANNEL f1 ========	
NUC1	1H
P0	11.60 usec
P1	11.60 usec
PL1	2.00 dB
SFO1	400.1317580 MHz
====== GRADIENT CHANNEL =====	
GPNAM1	SINE.100
GPZ1	10.00 %
P16	1000.00 usec
NDO	1
TD	128
SFO1	400.1318 MHz
FIDRES	25.040064 Hz
SW	8.010 ppm
FnMODE	QF
SI	1024
SF	400.1300473 MHz
WDW	SINE
SSB	0
LB	0.00 Hz
GB	0
PC	1.40
SI	1024
MC2	QF
SF	400.1300473 MHz
WDW	SINE
SSB	0
LB	0.00 Hz
GB	0

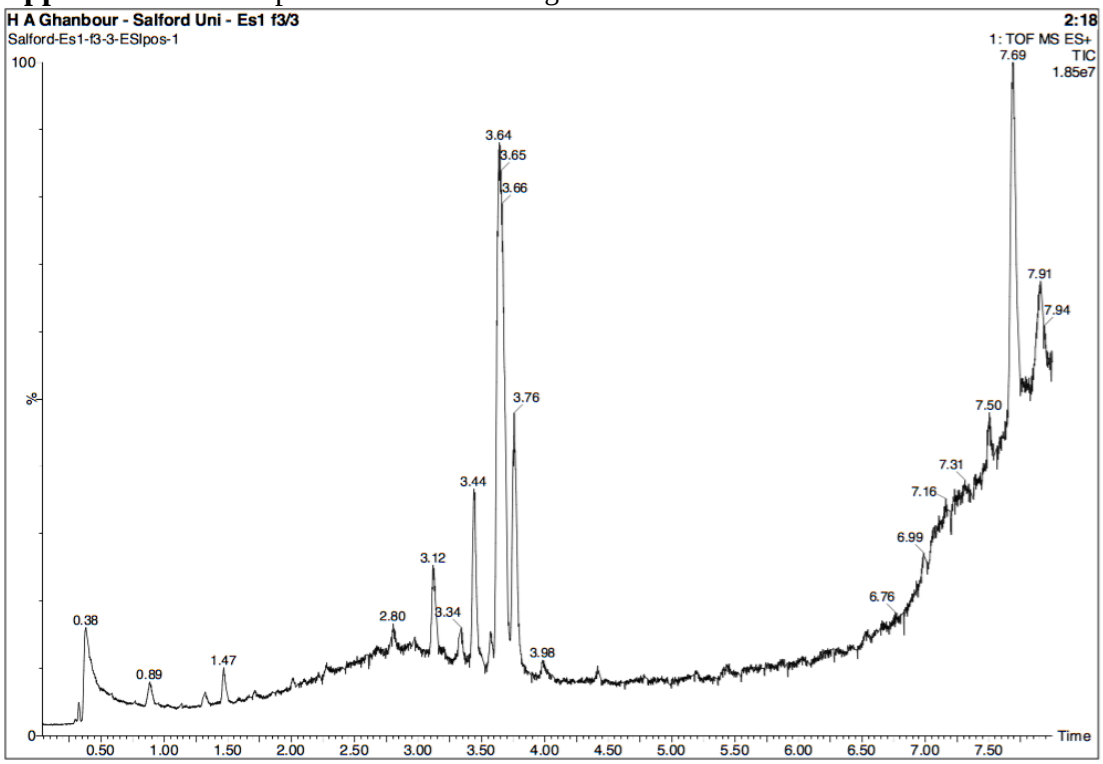
Appendix 3.23: BB 1H Decoupled 13C NMR Spectrum of F3/3 in D6DMSO, the signal observed was weak as limited amount of material was available.

Appendix 3.24: JMOD 13C NMR Spectrum of F3/3 in CD_3OD the signal observed was weak as limited amount of material was available

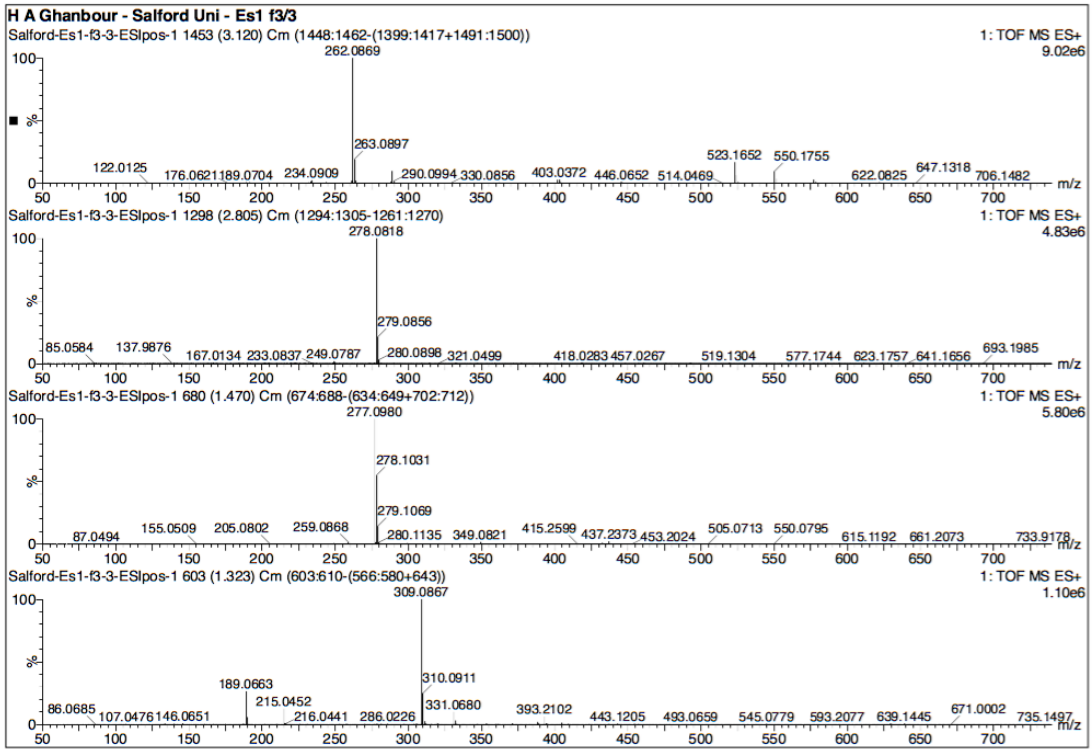
Appendix 3.25: The negative ion chromatogram from LC MS-TOF of F3/3



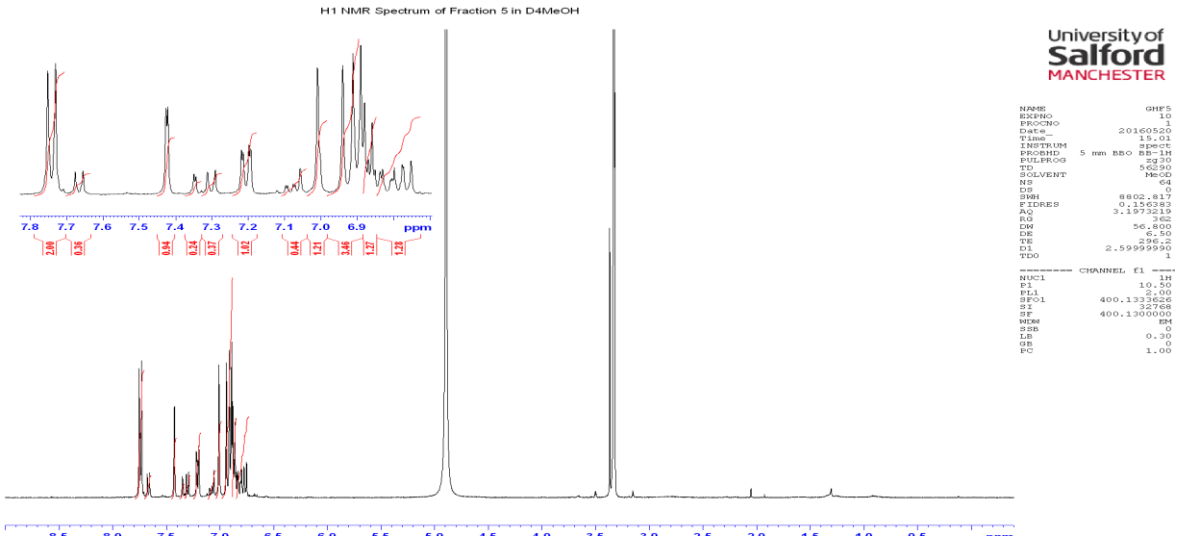
Appendix 3.26: The positive ion chromatogram from LC MS-TOF of F3/3



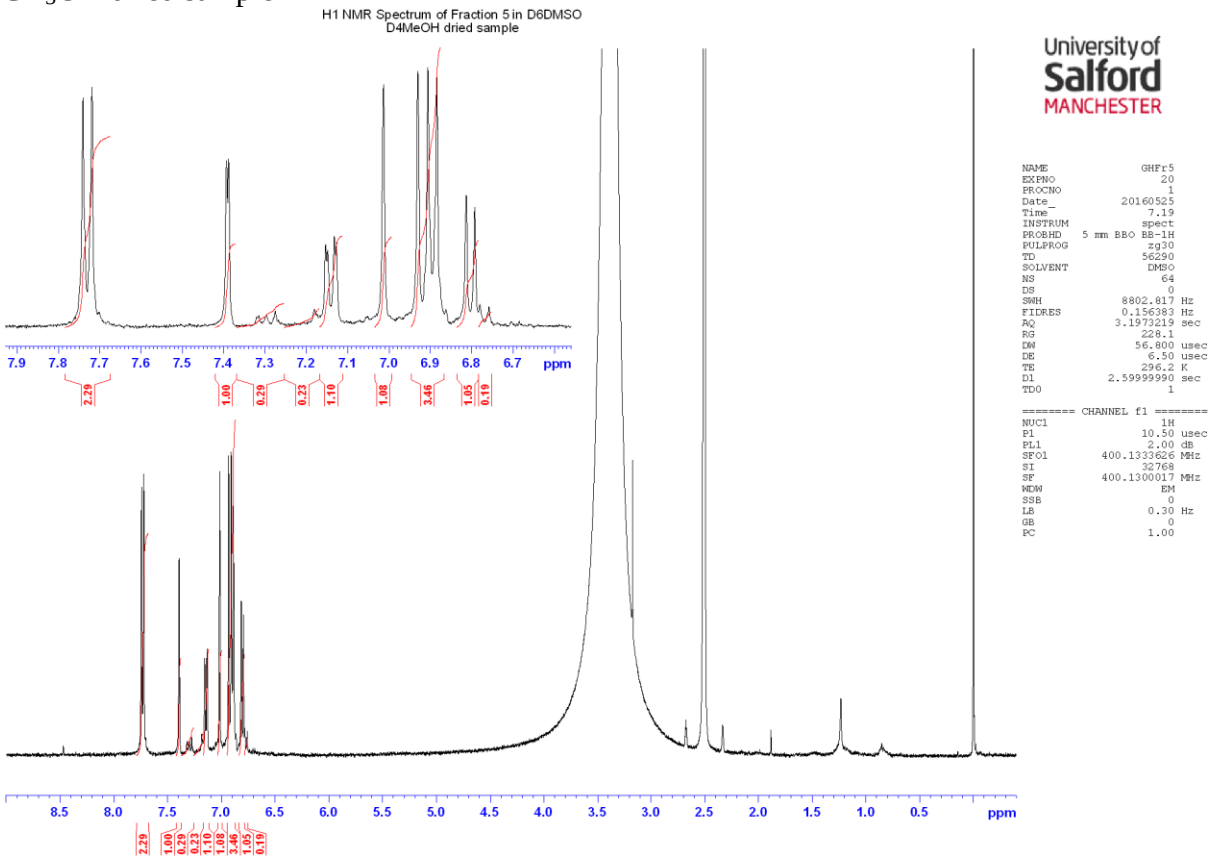
Appendix 3.27: The positive ion spectrum from LC MS-TOF of F3/3



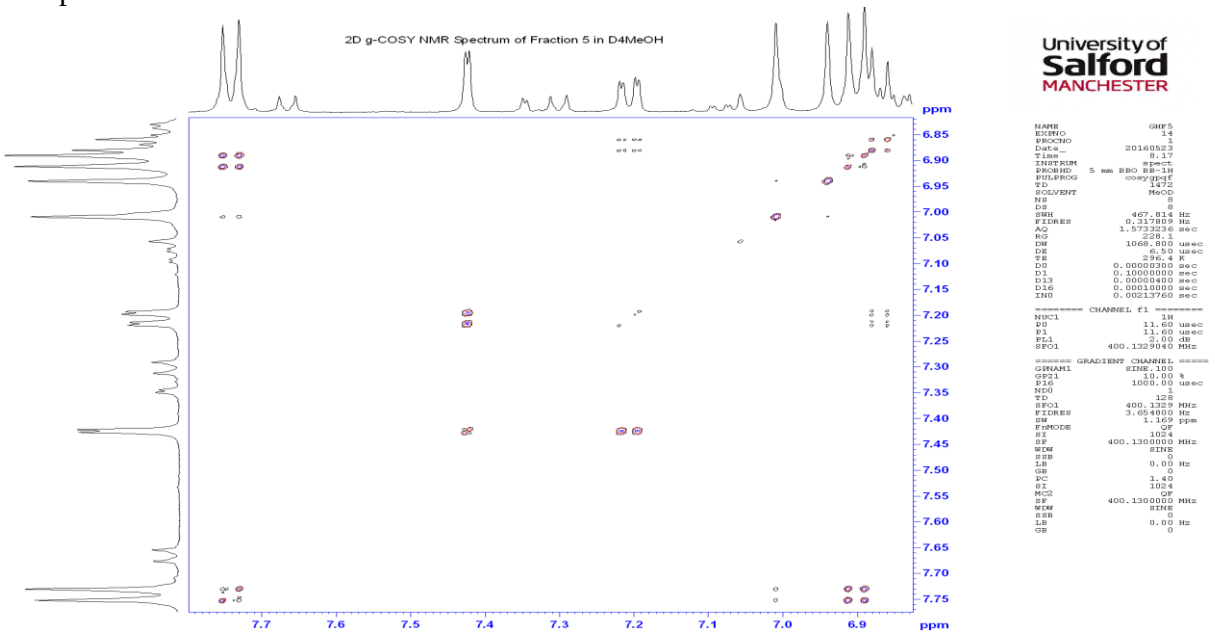
Appendix 3.28: ^{1}H NMR Spectrum of F5 in CD_3OD+TMS, the minor peaks observed in the above spectrum is due to degraded of the main component



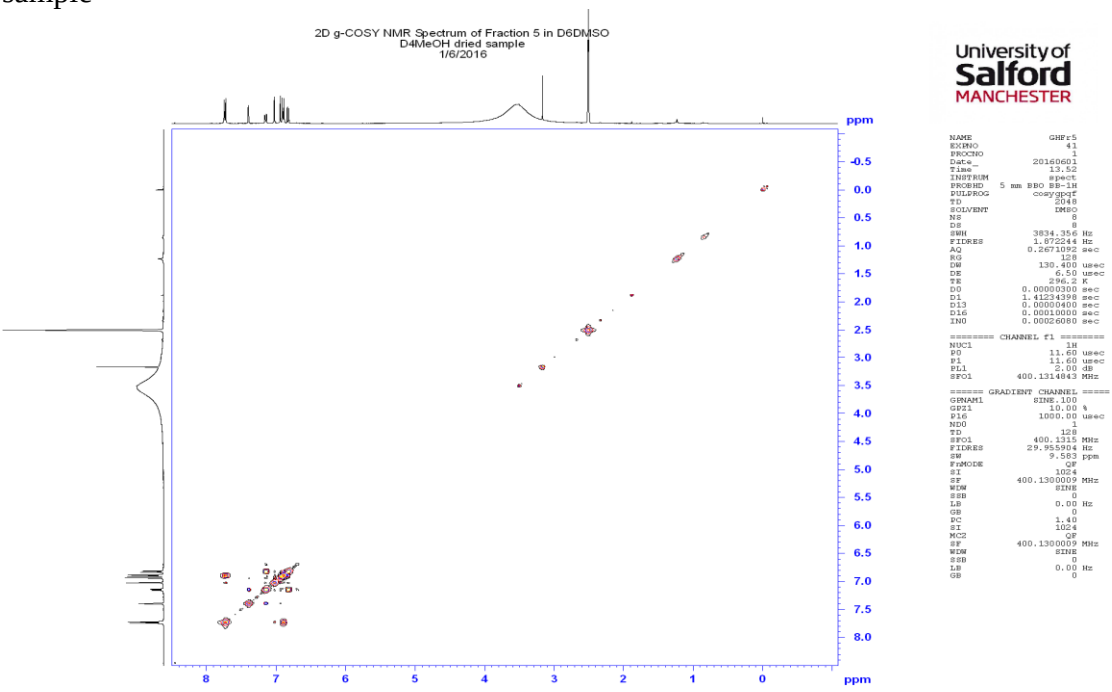
Appendix 3.29: ^{1}H NMR Spectrum of F5 in D_6DMSO+TMS in D6DMSO after CD_3OD dried sample



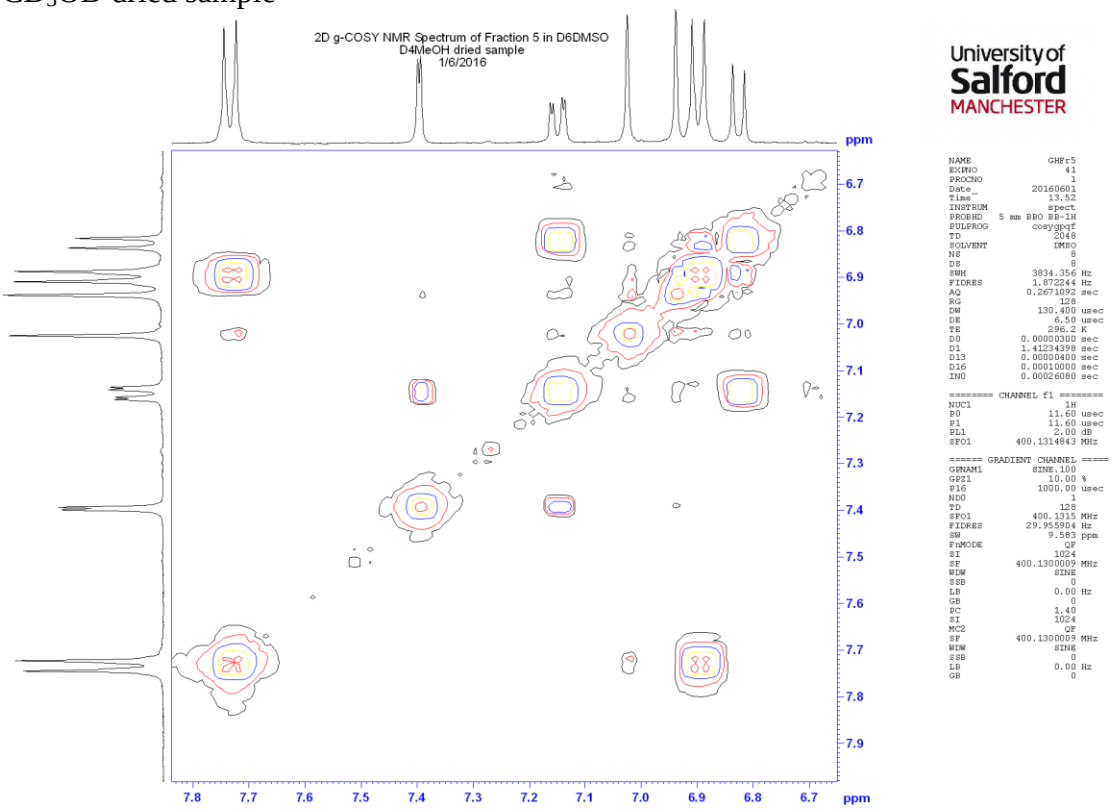
Appendix 3.30: 2D g-COSY NMR Spectrum of F5 (expanded) in CD_3OD, the minor peaks observed in the above spectrum are probably due to degraded of the main component



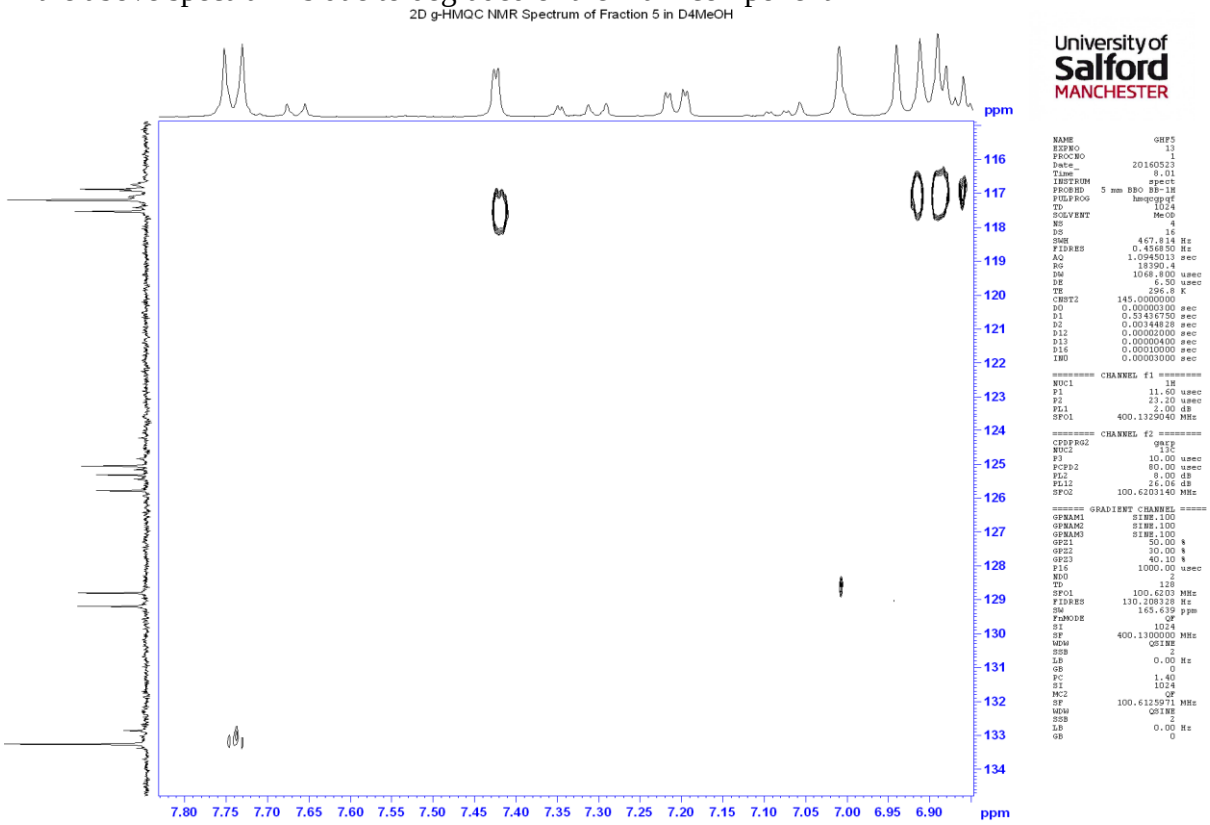
Appendix 3.31: 2D g-COSY NMR Spectrum of F5 in D6DMSO after CD_3OD dried sample



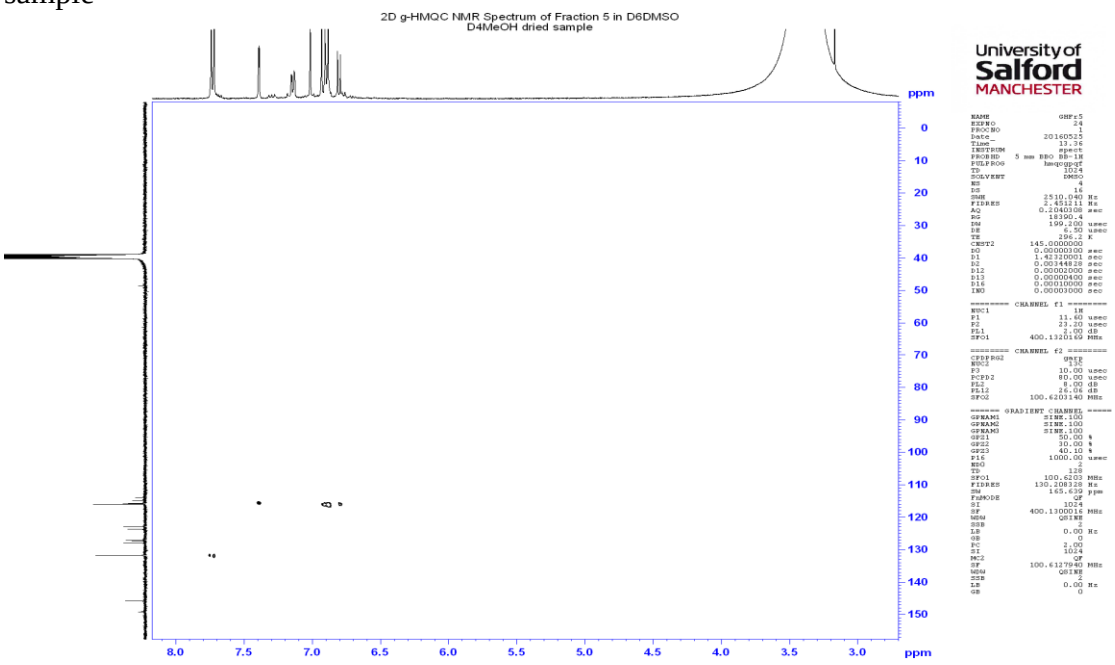
Appendix 3.32: 2D g-COSY NMR Spectrum of F5 (expansion) in D6DMSO after CD_3OD dried sample



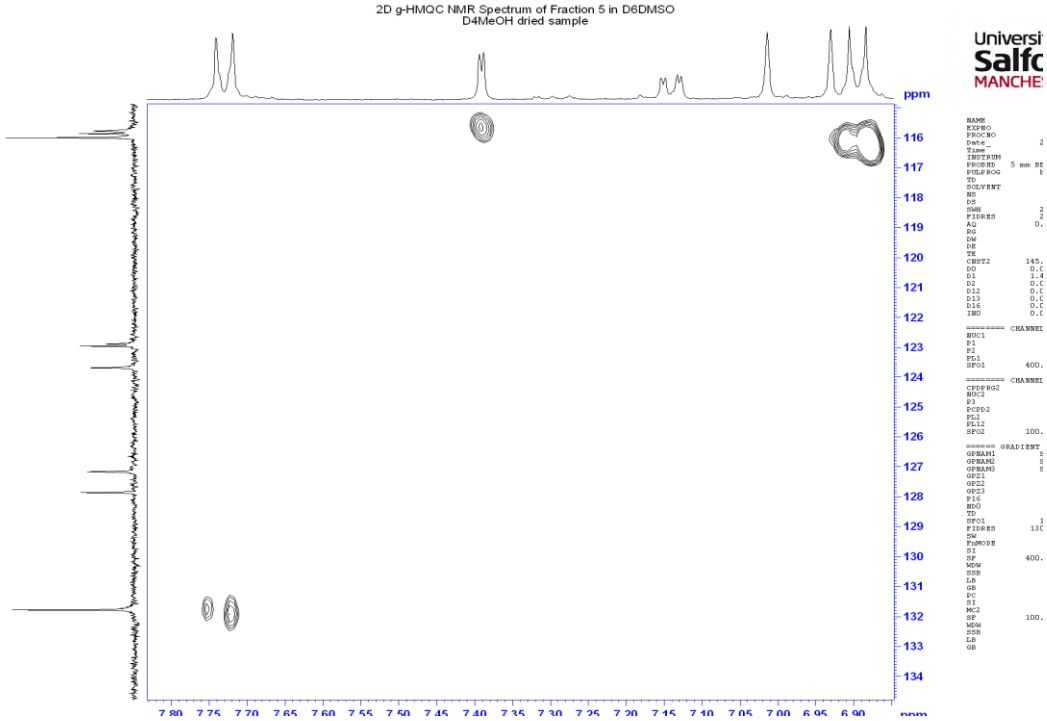
Appendix 3.33: 2D g-HMQC Spectrum of F5 in CD_3OD, the minor peaks observed in the above spectrum is due to degraded of the main component



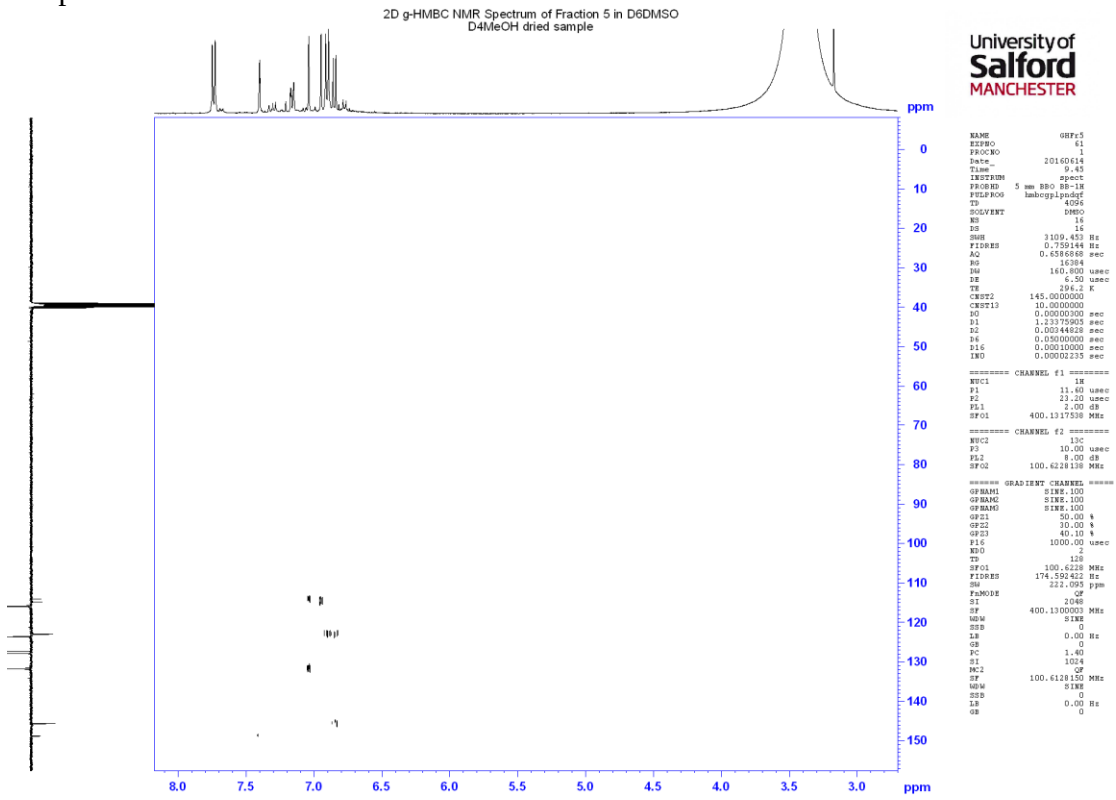
Appendix 3.34: 2D g-HMQC Spectrum of F5 in D6DMSO after CD_3OD dried sample



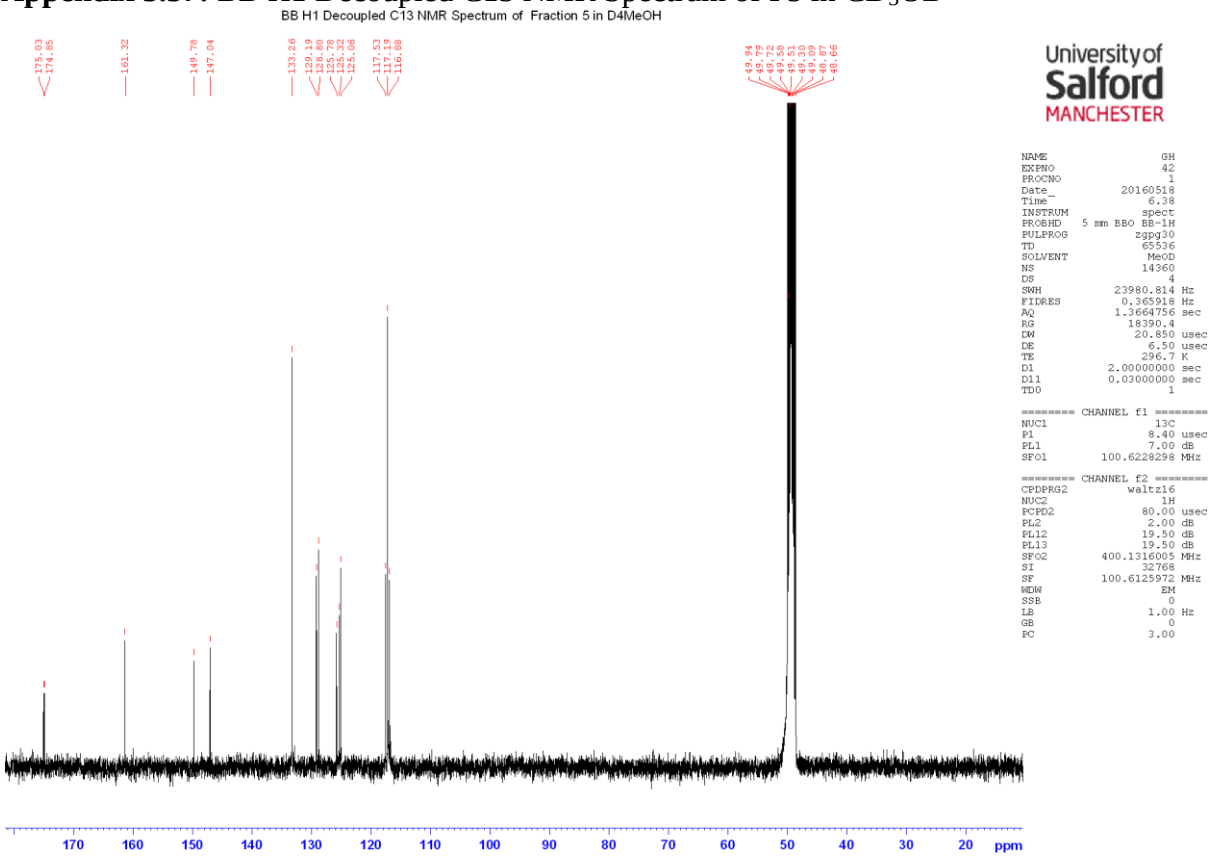
Appendix 3.35: 2D g-HMQC Spectrum of F5 (expansion) in D6DMSO after CD_3OD dried sample



Appendix 3.36: 2D g-HMBC Spectrum of F5 in D6DMSO after CD_3OD dried sample



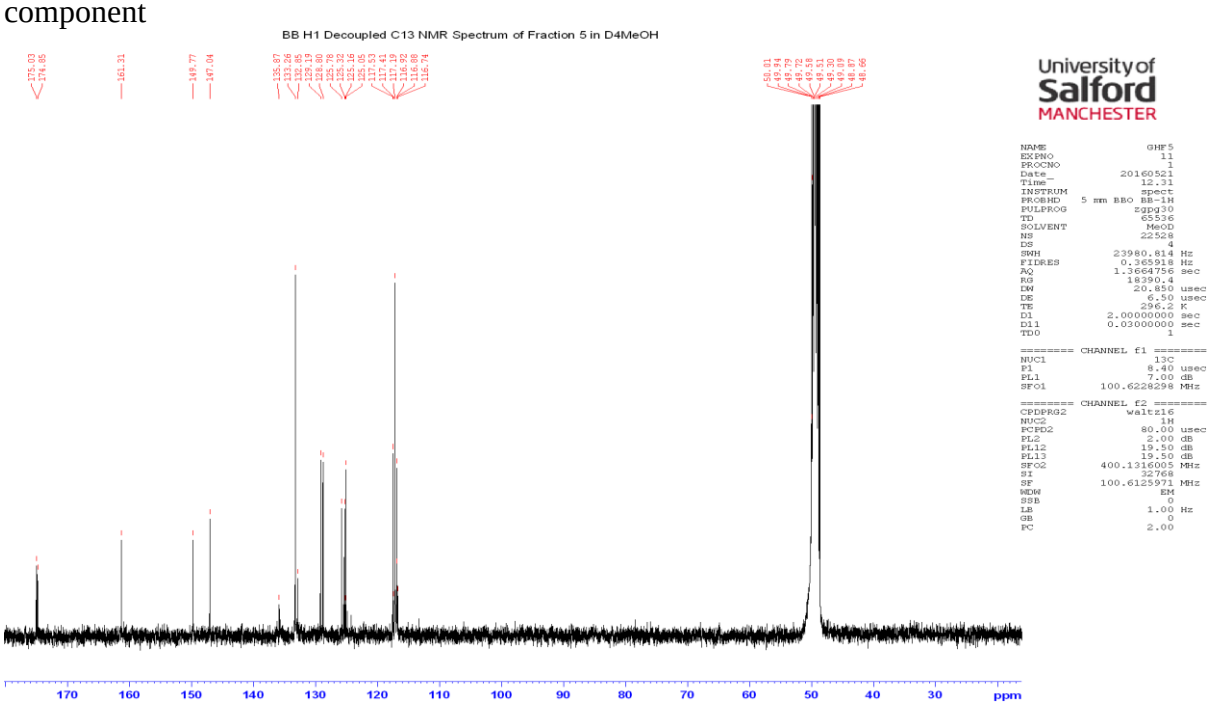
Appendix 3.37: BB H1 Decoupled C13 NMR Spectrum of F5 in CD_3OD



University of Salford MANCHESTER

NAME	GH
EXPNO	42
PROCNO	1
Date_	20160518
Time	6.38
INSTRUM	spect
PROBHD	5 mm BBO BB-1H
PULPROG	zgpg30
TD	65536
SOLVENT	MeOD
NS	14360
DS	4
SWH	23980.814 Hz
FIDRES	0.365918 Hz
AQ	1.3664756 sec
RG	18390.4
DW	20.850 usec
DE	6.50 usec
TE	296.7 K
D1	2.00000000 sec
D11	0.03000000 sec
TD0	1
======== CHANNEL f1 ========	
NUC1	13C
P1	8.40 usec
PL1	7.00 dB
SFO1	100.6228298 MHz
======== CHANNEL f2 ========	
CPDPRG2	waltz16
NUC2	1H
PCPD2	80.00 usec
PL2	2.00 dB
PL12	19.50 dB
PL13	19.50 dB
SFO2	400.1316005 MHz
SI	32768
SF	100.6125972 MHz
WDW	EM
SSB	0
LB	1.00 Hz
GB	0
PC	3.00

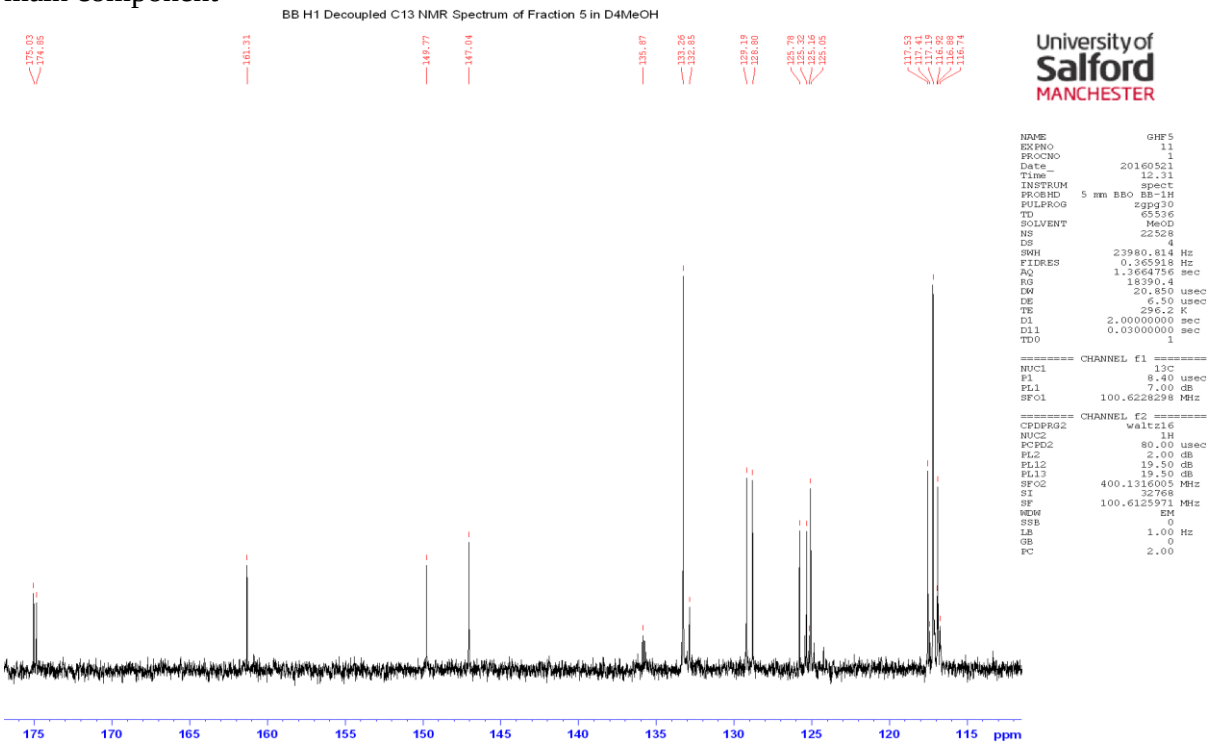
Appendix 3.38: BB H1 Decoupled C13 NMR Spectrum of F5 in CD_3OD, after the minor peaks observed in the proton spectrum of F5due to degraded of the main component



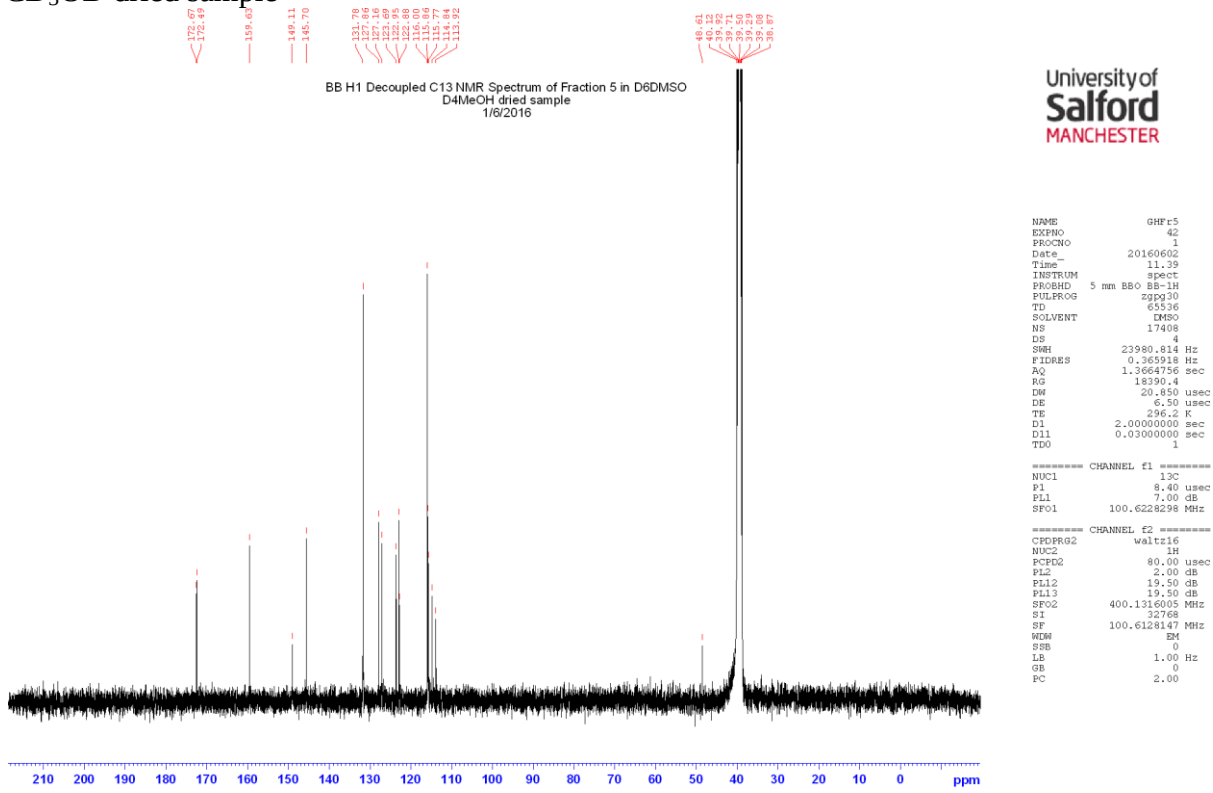
University of Salford MANCHESTER

NAME	GHF5
EXPNO	11
PROCNO	1
Date_	20160521
Time	12.31
INSTRUM	spect
PROBHD	5 mm BBO BB-1H
PULPROG	zgpg30
TD	65536
SOLVENT	MeOD
NS	22528
DS	4
SWH	23980.814 Hz
FIDRES	0.365918 Hz
AQ	1.3664756 sec
RG	18390.4
DW	20.850 usec
DE	6.50 usec
TE	296.2 K
D1	2.00000000 sec
D11	0.03000000 sec
TD0	1
======== CHANNEL f1 ========	
NUC1	13C
P1	8.40 usec
PL1	7.00 dB
SFO1	100.6228298 MHz
======== CHANNEL f2 ========	
CPDPRG2	waltz16
NUC2	1H
PCPD2	80.00 usec
PL2	2.00 dB
PL12	19.50 dB
PL13	19.50 dB
SFO2	400.1316005 MHz
SI	32768
SF	100.6125971 MHz
WDW	EM
SSB	0
LB	1.00 Hz
GB	0
PC	2.00

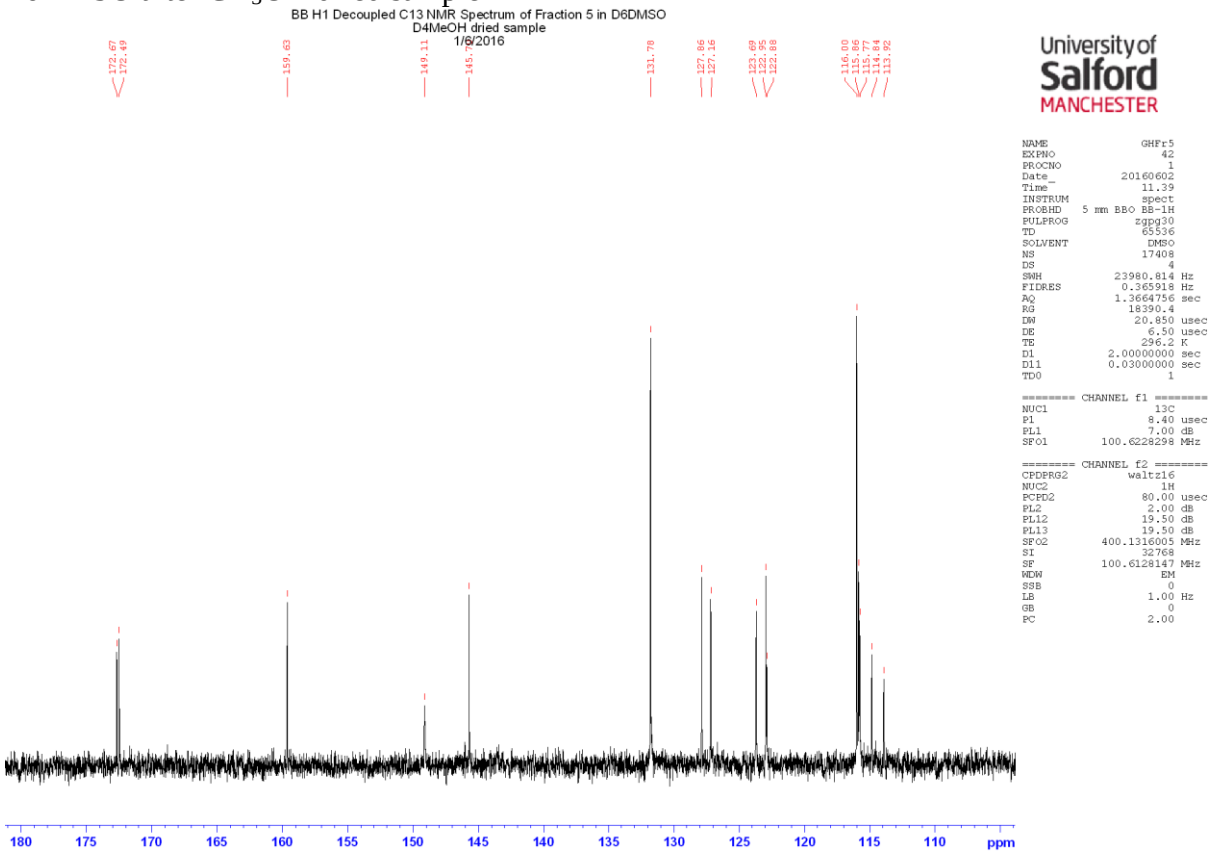
Appendix 3.39: BB H1 Decoupled C13 NMR Spectrum of F5 (expanded) in CD_3OD, after the minor peaks observed in the proton spectrum of F5 due to degraded of the main component



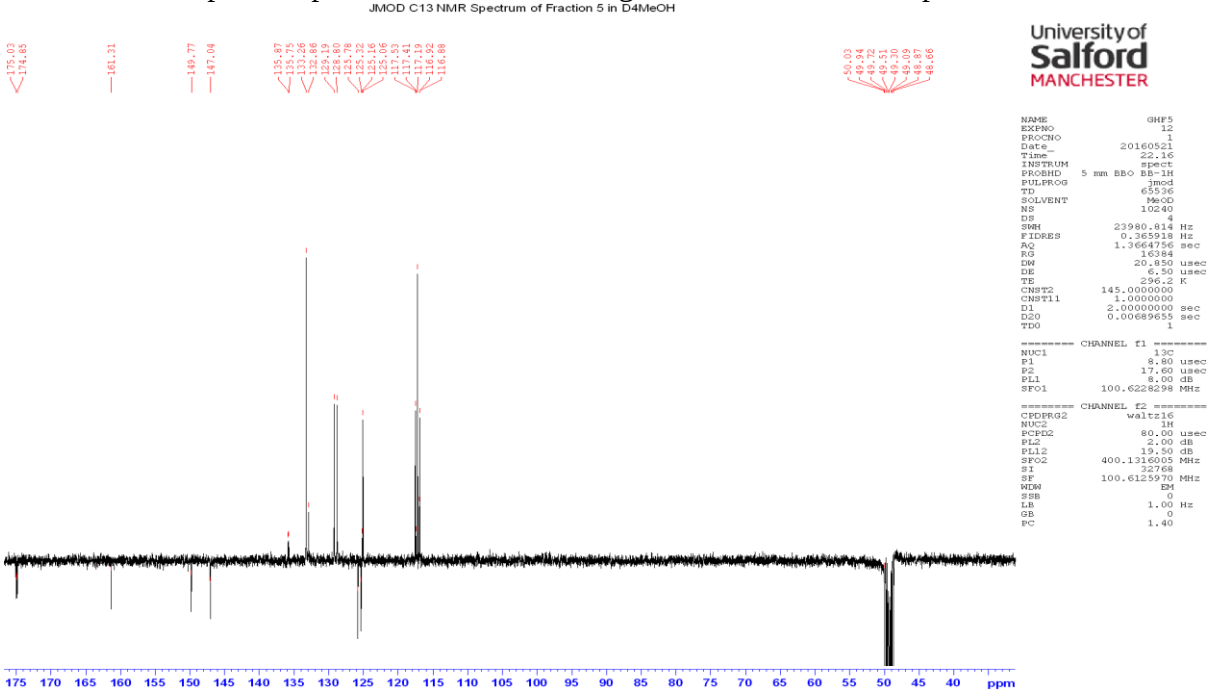
Appendix 3.40: BB H1 Decoupled C13 NMR Spectrum of F5 in D6DMSO after CD_3OD dried sample



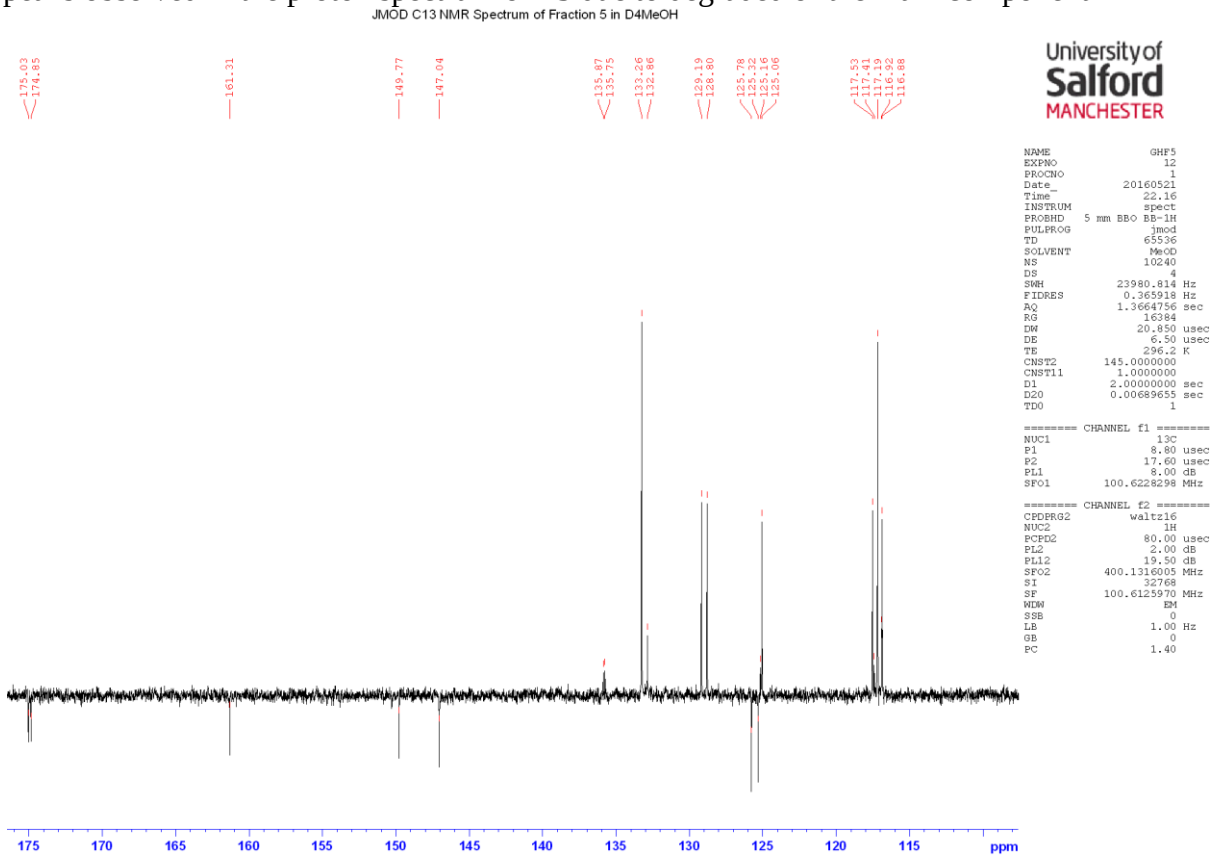
Appendix 3.41: BB H1 Decoupled C13 NMR Spectrum of F5 (expansion) in D6DMSO after CD_3OD dried sample



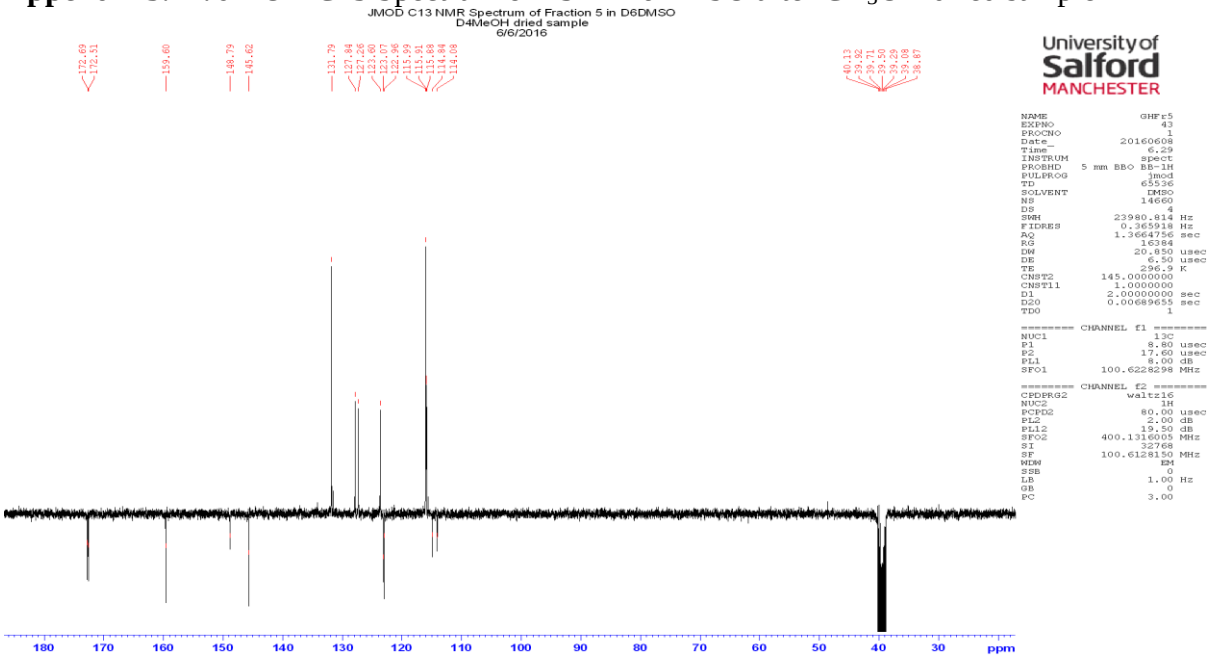
Appendix 3.42: JMOD C13 Spectrum of F5 in CD_3OD, after the minor peaks observed in the proton spectrum of F5 due to degraded of the main component



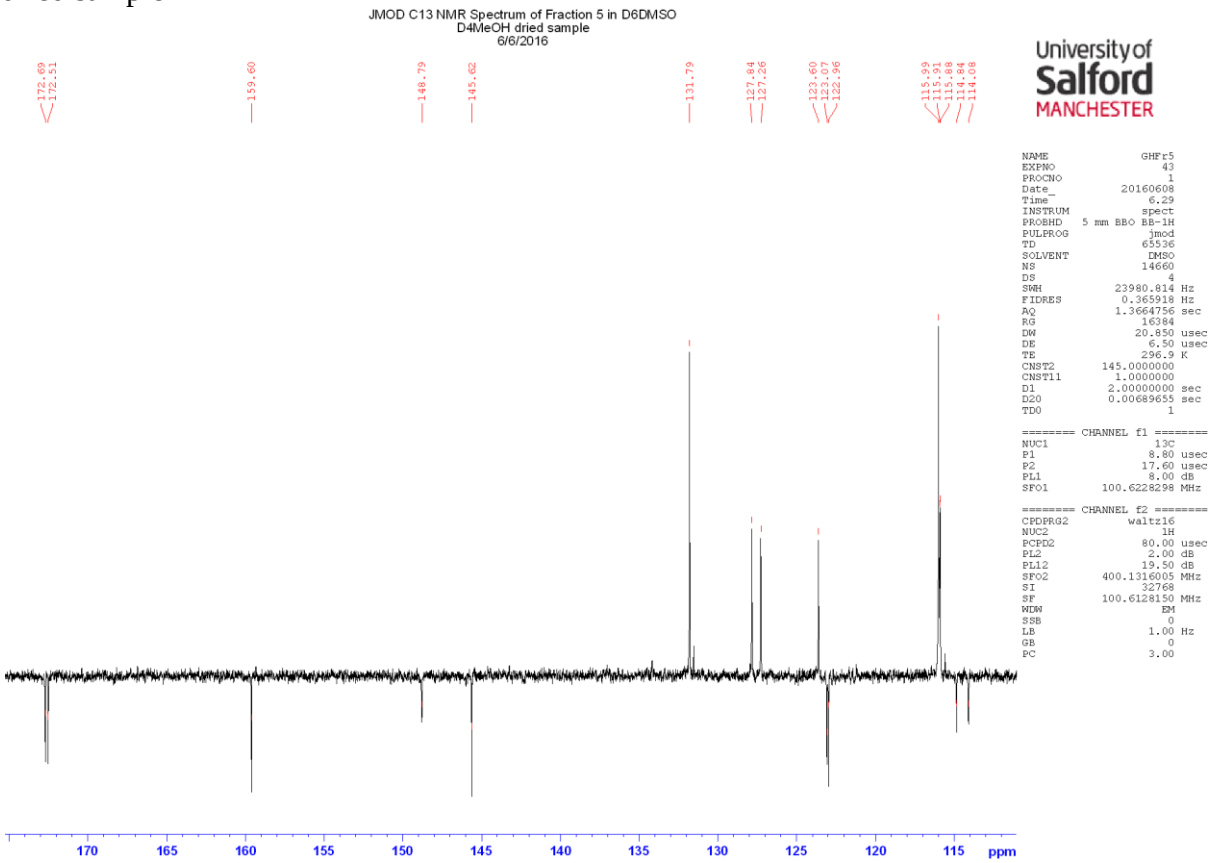
Appendix 3.43: JMOD C13 Spectrum of F5 (expansion) in CD_3OD, after the minor peaks observed in the proton spectrum of F5 due to degraded of the main component



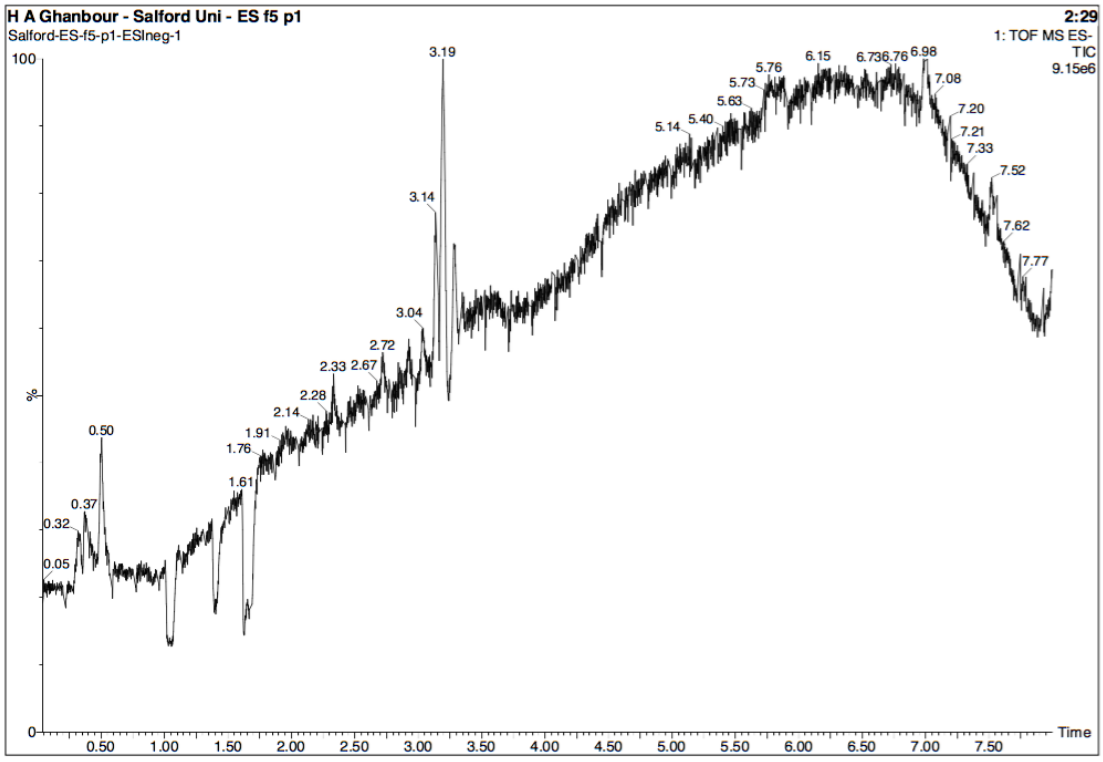
Appendix 3.44: JMOD C13 Spectrum of F5 in D6DMSO after CD_3OD dried sample



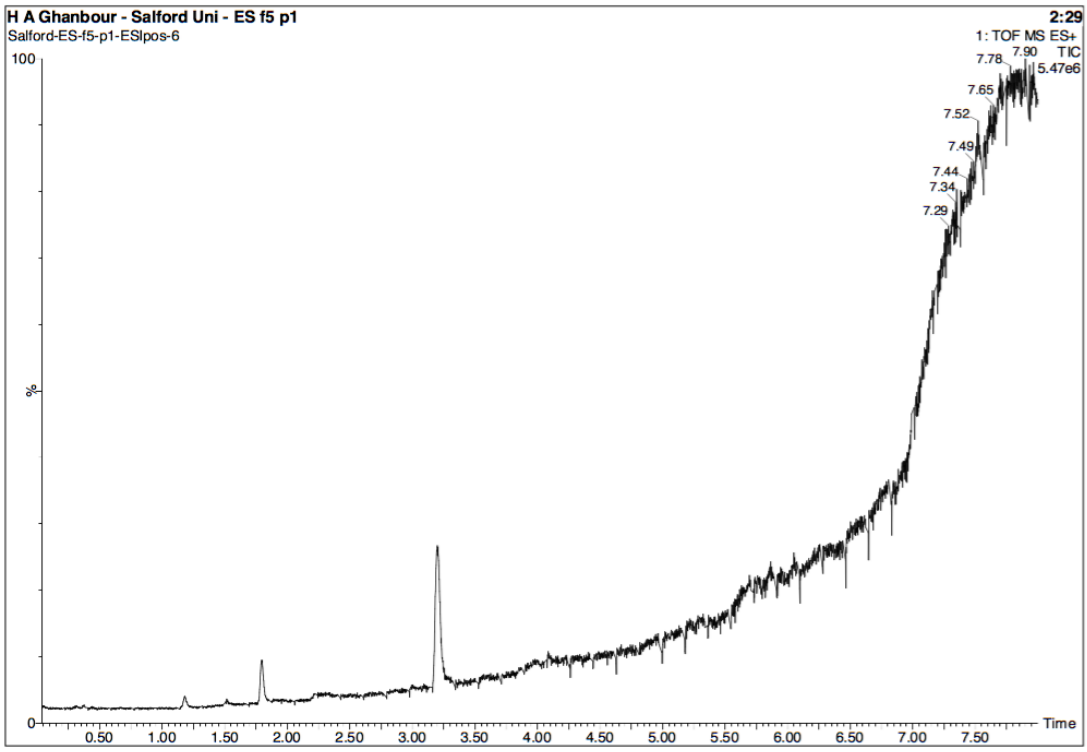
Appendix 3.45: JMOD C13 Spectrum of F5 (expansion) in D6DMSO after CD_3OD dried sample



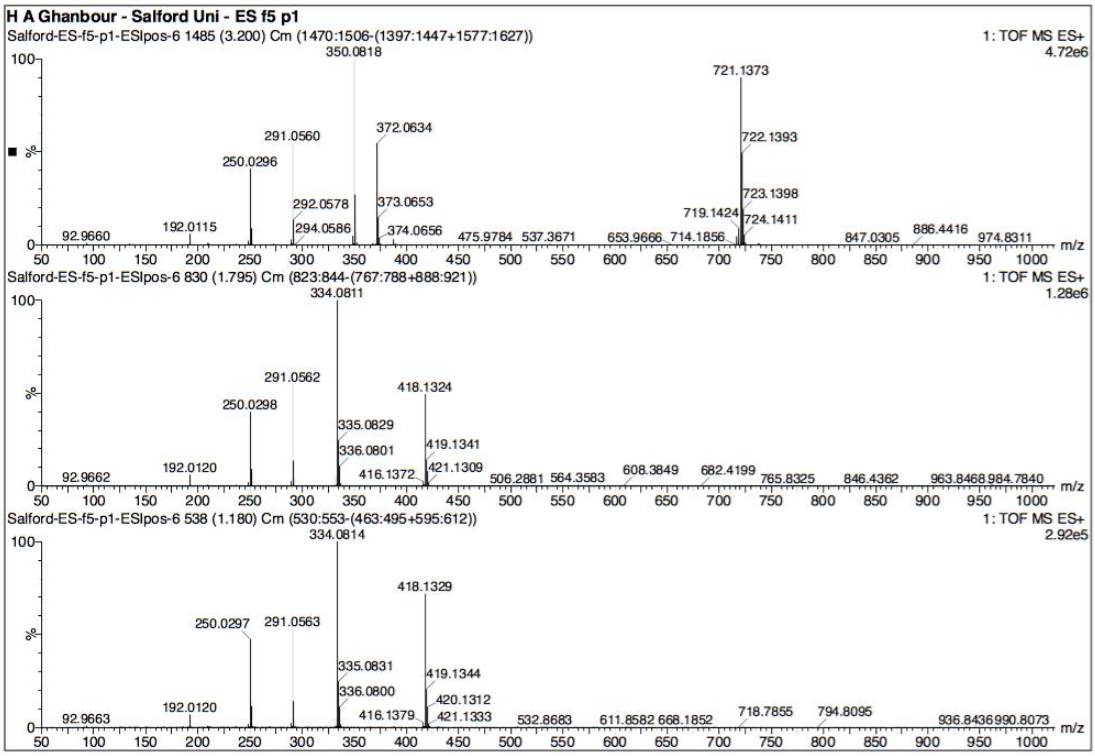
Appendix 3.46: The negative ion chromatogram from LC MS-TOF of F5



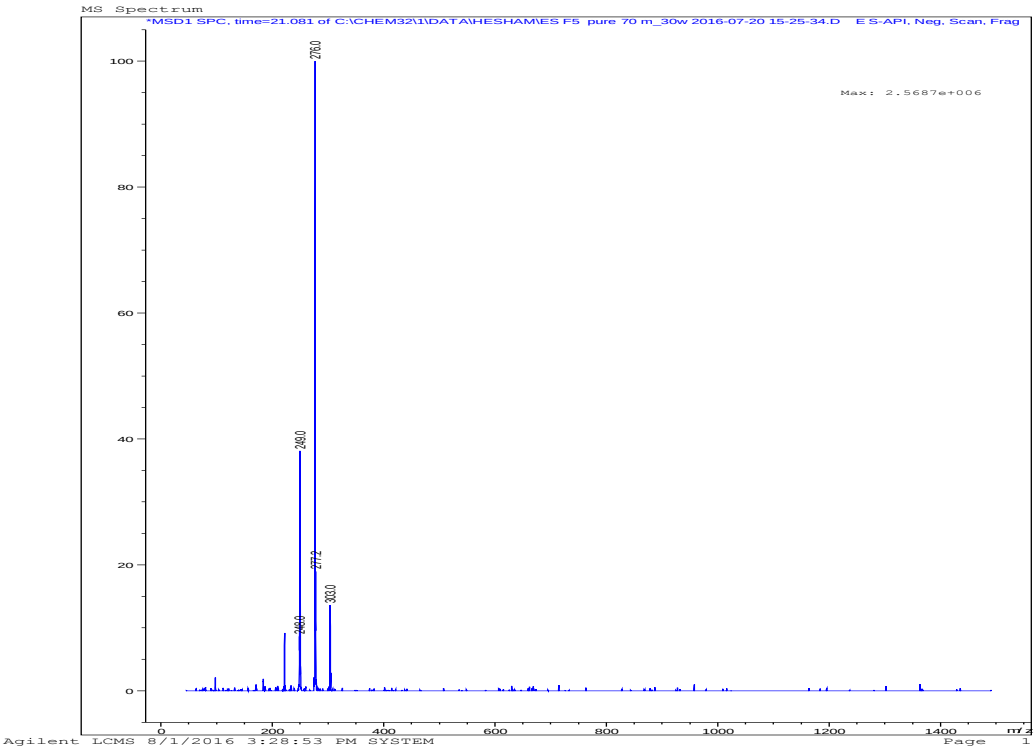
Appendix 3.47: The positive ion chromatogram from LC MS-TOF of F5



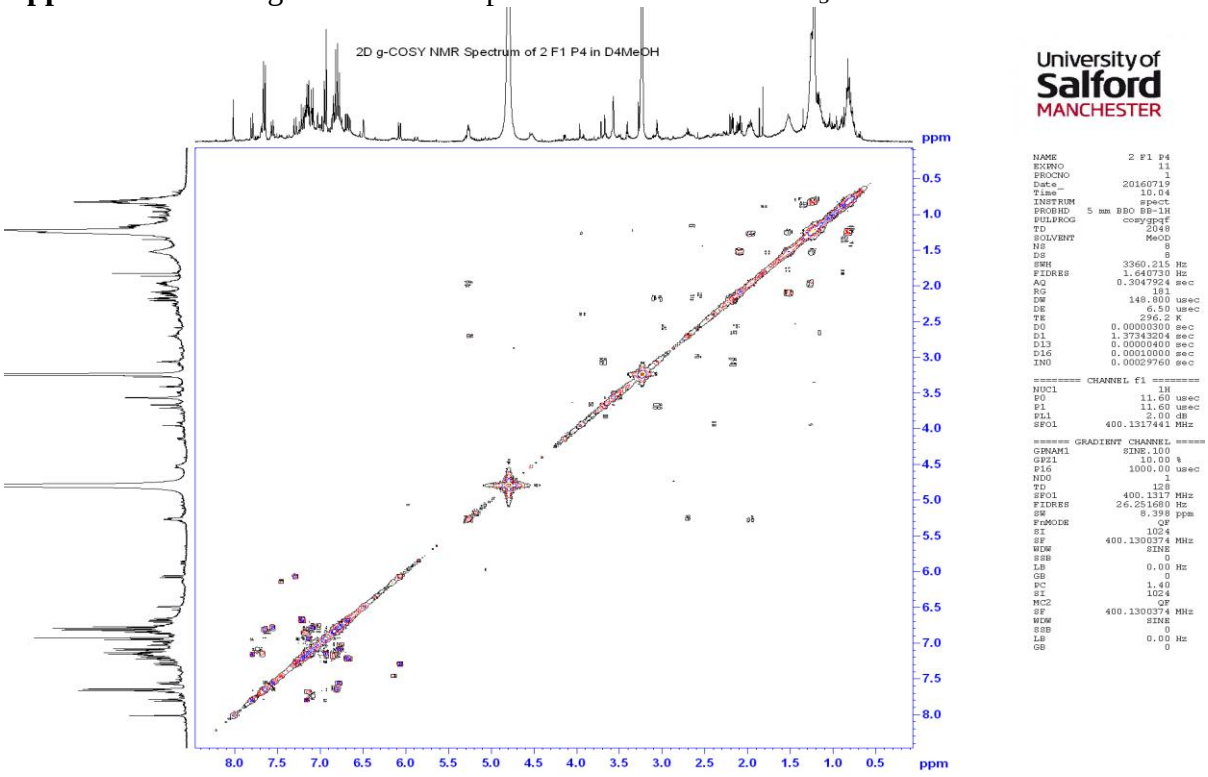
Appendix 3.48: The positive ion spectrum from LC MS-TOF of F5



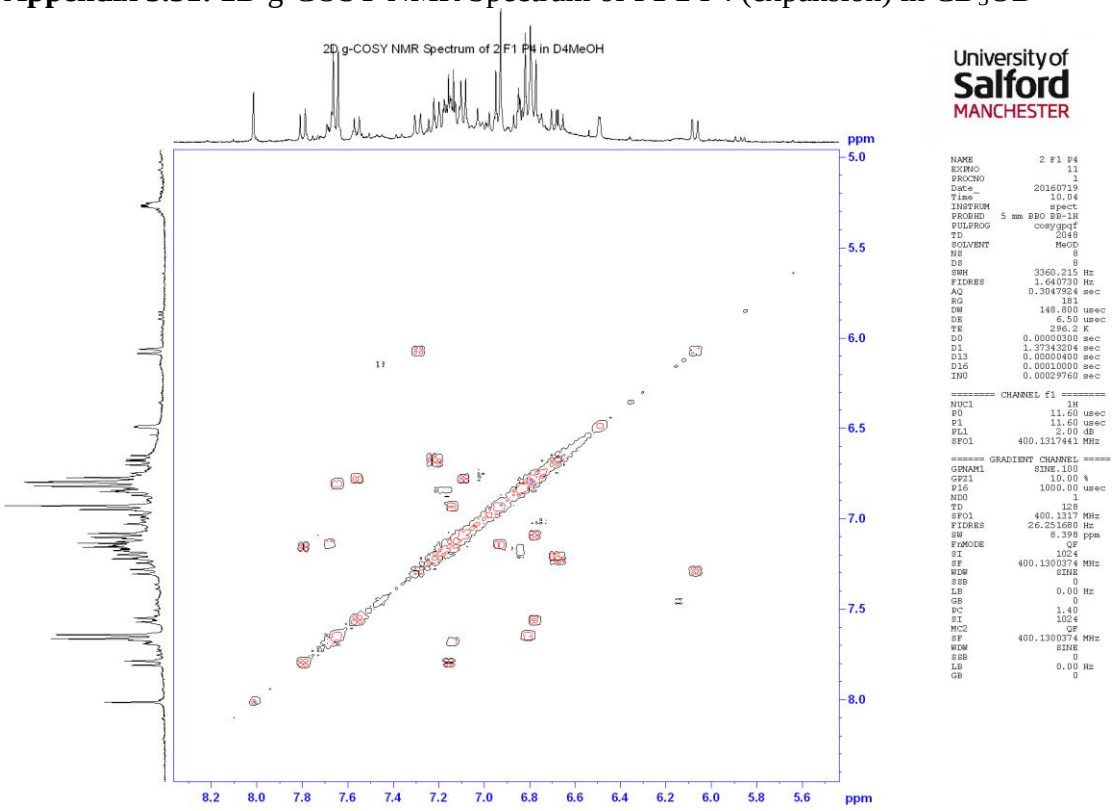
Appendix 3.49: The negative ion spectrum from LC MS (Salford University) of F5



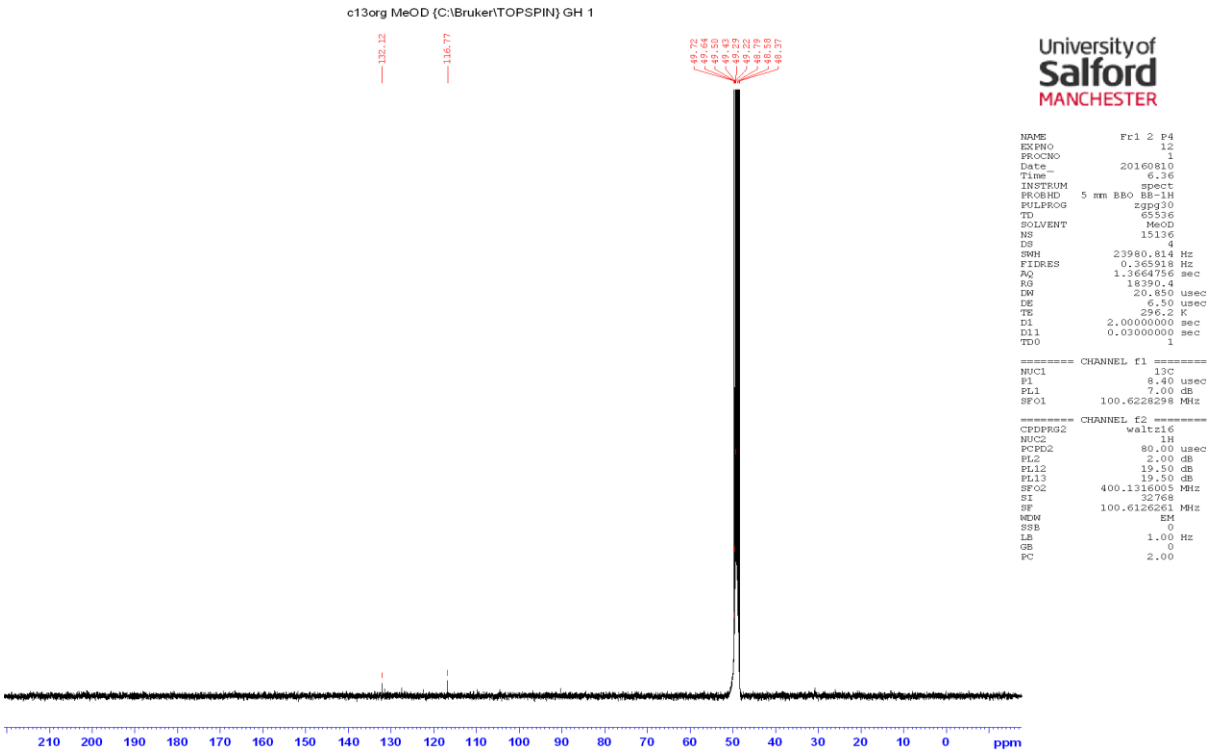
Appendix 3.50: 2D g-COSY NMR Spectrum of F1 2 P4 in CD_3OD



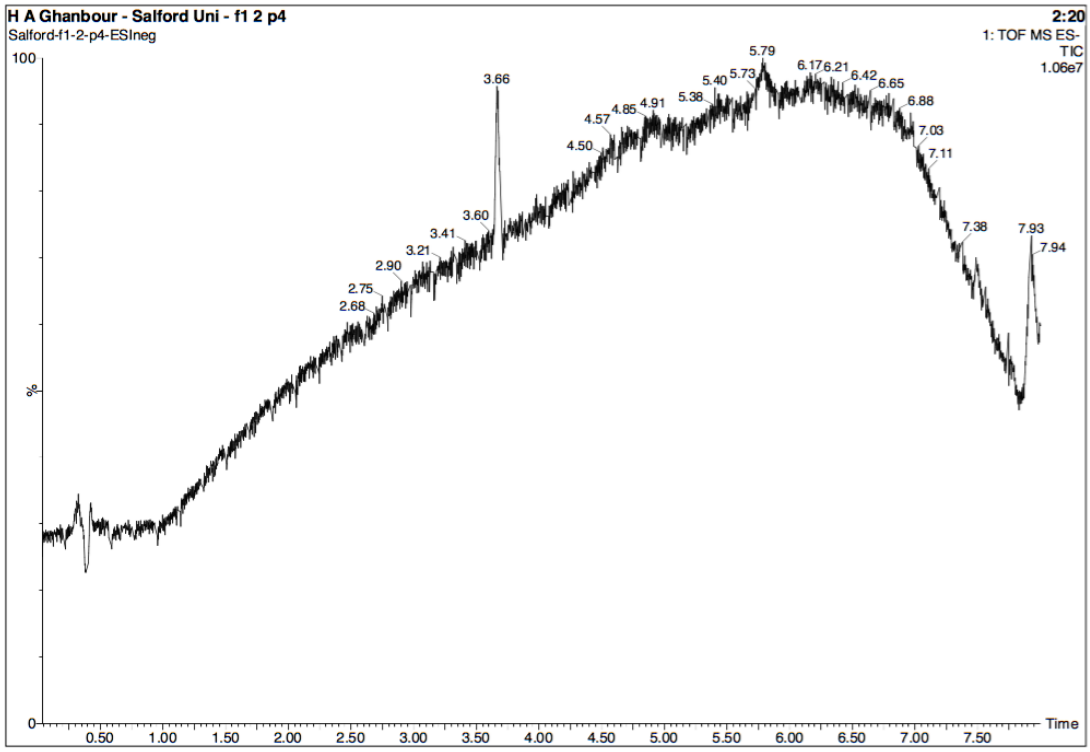
Appendix 3.51: 2D g-COSY NMR Spectrum of F1 2 P4 (expansion) in CD_3OD



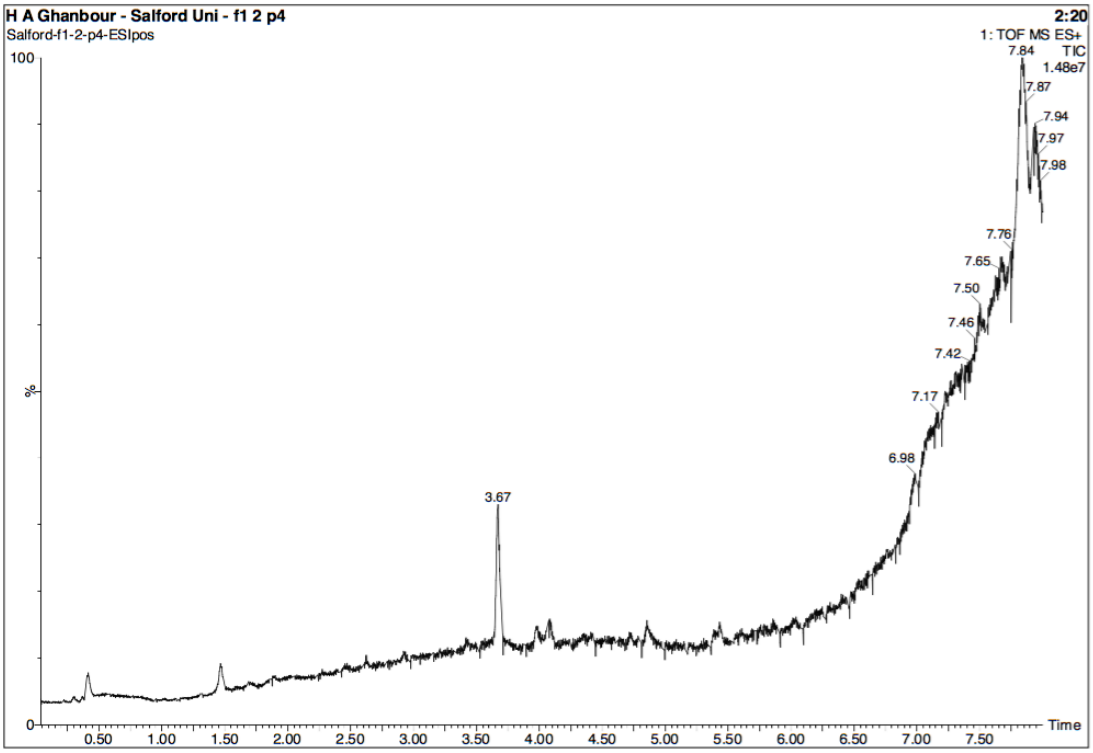
Appendix 3.52: BB H1 Decoupled C13 NMR Spectrum of F1 2 p4 in CD_3OD, the signal observed is weak as limited amount of material was available



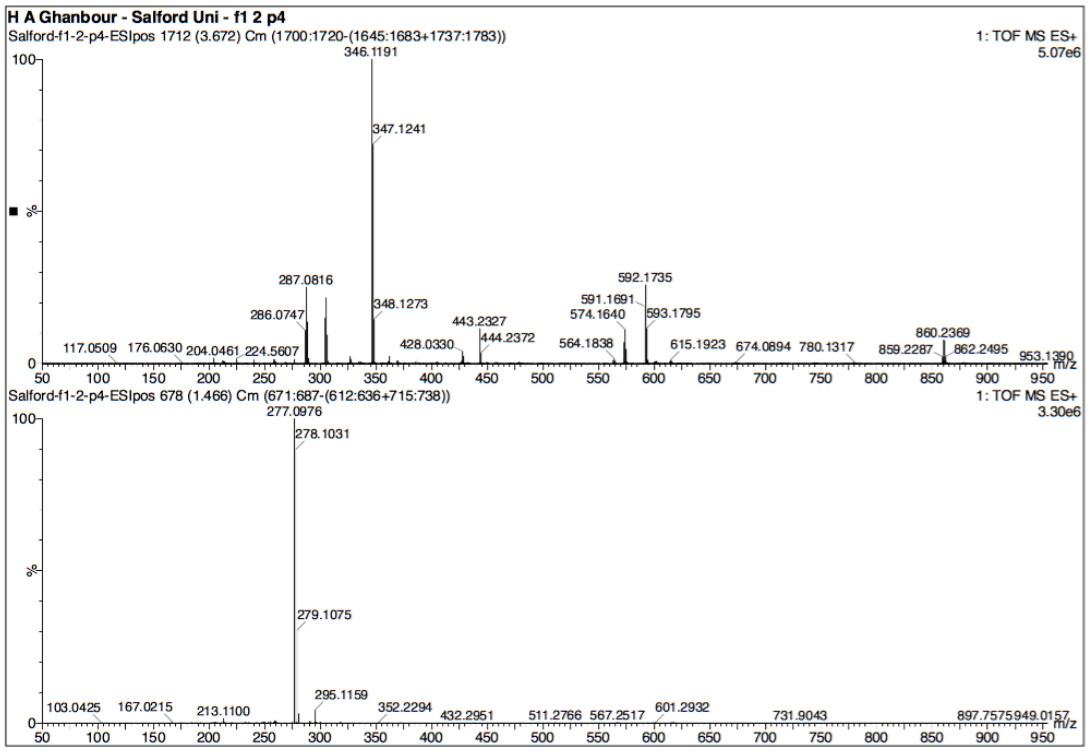
Appendix 3.53: The negative ion chromatogram from LC MS-TOF of F1 2 p4



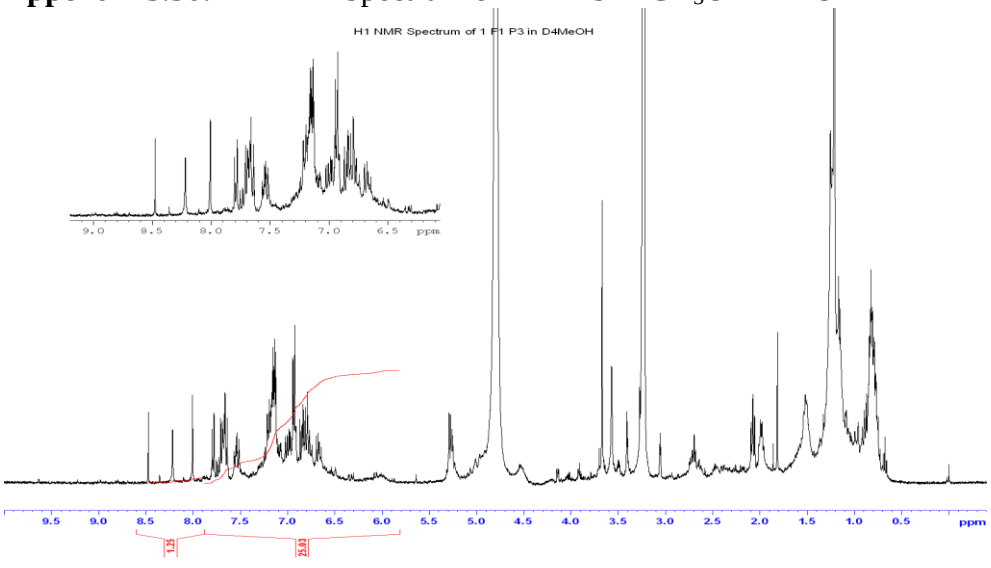
Appendix 3.54: The positive ion chromatogram from LC MS-TOF of F1 2 p4



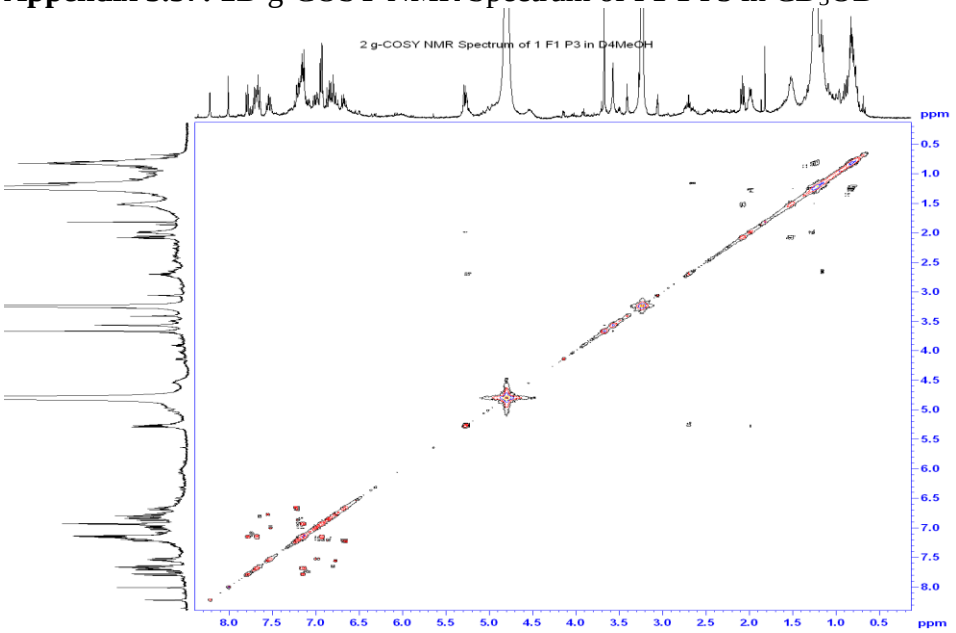
Appendix 3.55: The positive ion spectrum from LC MS-TOF of F1 2 p4



Appendix 3.56: ^{1}H NMR Spectrum of F1 1 P3 in CD_3OD+TMS



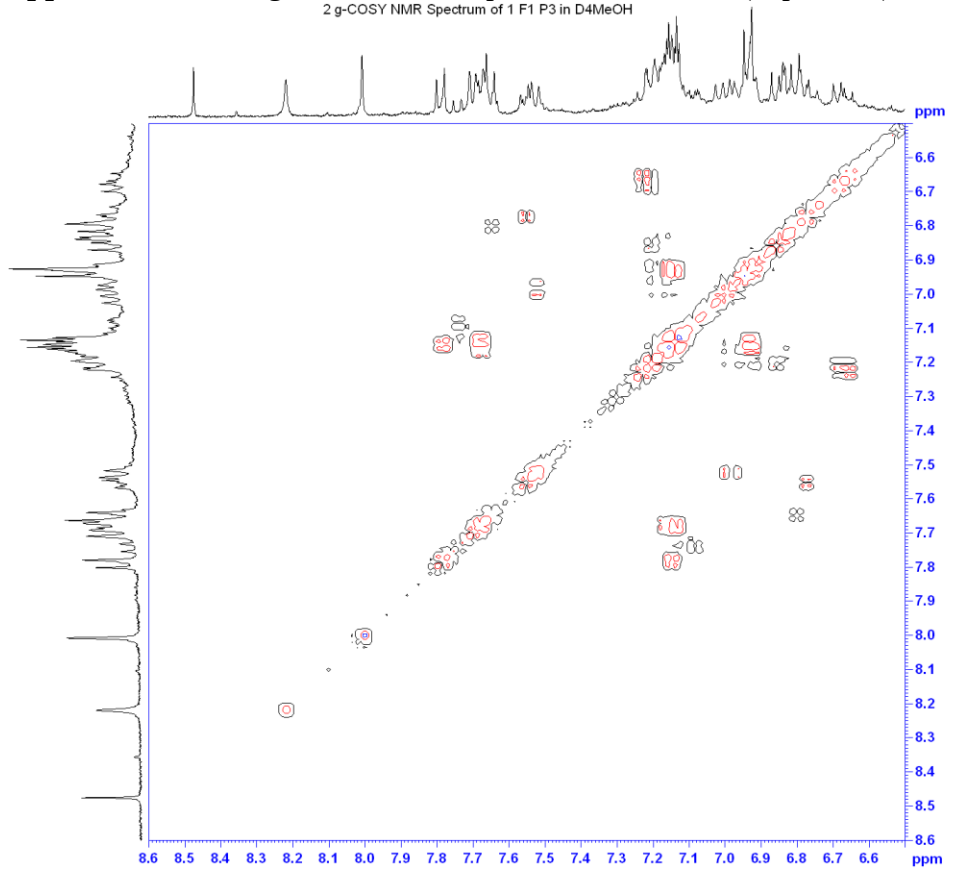
Appendix 3.57: 2D g-COSY NMR Spectrum of F1 1 P3 in CD_3OD



University of Salford MANCHESTER

```
NAME                  1 F1 P3
EXPNO                      11
PROCNO                      1
Date_              20160719
Time                    9.29
INSTRUM                 spect
PROBHD    5 mm BBO BB-1H
PULPROG             cosygpqf
TD                      2048
SOLVENT                 MeOD
NS                         8
DS                         8
SWH                3306.878 Hz
FIDRES             1.614687 Hz
AQ                 0.3097076 sec
RG                     161.3
DW                   151.200 usec
DE                      6.50 usec
TE                     296.2 K
D0                 0.00000300 sec
D1                 1.36933601 sec
D13                0.00000400 sec
D16                0.00010000 sec
IN0                0.00030240 sec

======== CHANNEL f1 ========
NUC1                       1H
P0                      11.60 usec
P1                      11.60 usec
PL1                      2.00 dB
SFO1               400.1317429 MHz

====== GRADIENT CHANNEL =====
GPNAM1                SINE.100
GPZ1                     10.00 %
P16                    1000.00 usec
NDO                        1
TD                       128
SFO1               400.1317 MHz
FIDRES              25.834988 Hz
SW                       8.264 ppm
FnMODE                    QF
SI                       1024
SF                 400.1300374 MHz
WDW                      SINE
SSB                         0
LB                       0.00 Hz
GB                          0
PC                       1.40
SI                       1024
MC2                       QF
SF                 400.1300374 MHz
WDW                      SINE
SSB                         0
LB                       0.00 Hz
GB                          0
```

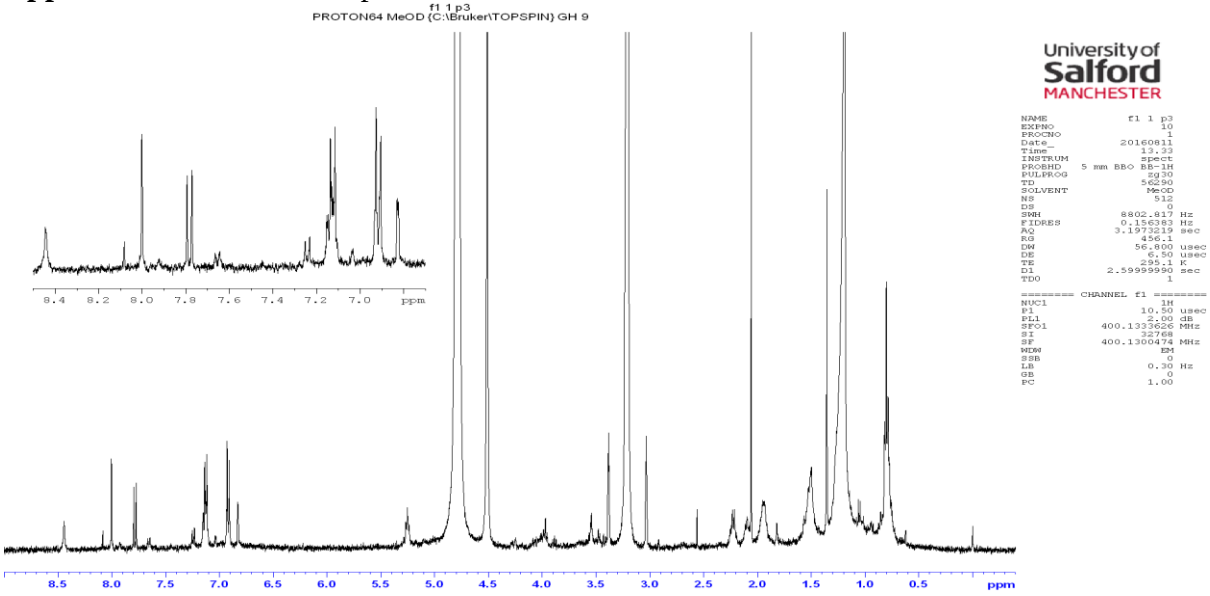
Appendix 3.58: 2D g-COSY NMR Spectrum of F1 1 P3 (expansion) in CD_3OD



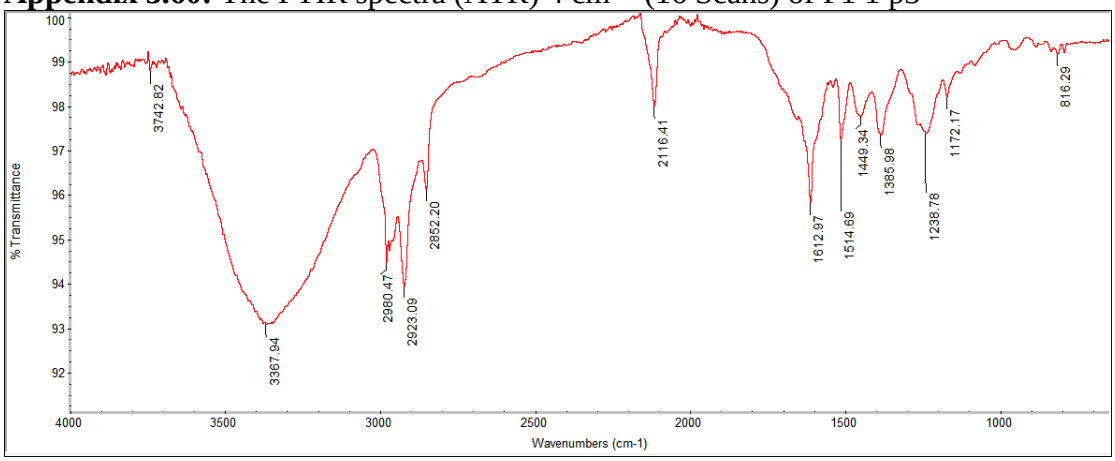
University of Salford MANCHESTER

```
NAME                  1 F1 P3
EXPNO                      11
PROCNO                      1
Date_              20160719
Time                    9.29
INSTRUM                 spect
PROBHD    5 mm BBO BB-1H
PULPROG             cosygpqf
TD                      2048
SOLVENT                 MeOD
NS                         8
DS                         8
SWH                3306.878 Hz
FIDRES             1.614687 Hz
AQ                 0.3097076 sec
RG                     161.3
DW                   151.200 usec
DE                      6.50 usec
TE                     296.2 K
D0                 0.00000300 sec
D1                 1.36933601 sec
D13                0.00000400 sec
D16                0.00010000 sec
IN0                0.00030240 sec

======== CHANNEL f1 ========
NUC1                       1H
P0                      11.60 usec
P1                      11.60 usec
PL1                      2.00 dB
SFO1               400.1317429 MHz

====== GRADIENT CHANNEL =====
GPNAM1                SINE.100
GPZ1                     10.00 %
P16                    1000.00 usec
NDO                        1
TD                       128
SFO1               400.1317 MHz
FIDRES              25.834988 Hz
SW                       8.264 ppm
FnMODE                    QF
SI                       1024
SF                 400.1300374 MHz
WDW                      SINE
SSB                         0
LB                       0.00 Hz
GB                          0
PC                       1.40
SI                       1024
MC2                       QF
SF                 400.1300374 MHz
WDW                      SINE
SSB                         0
LB                       0.00 Hz
GB                          0
```

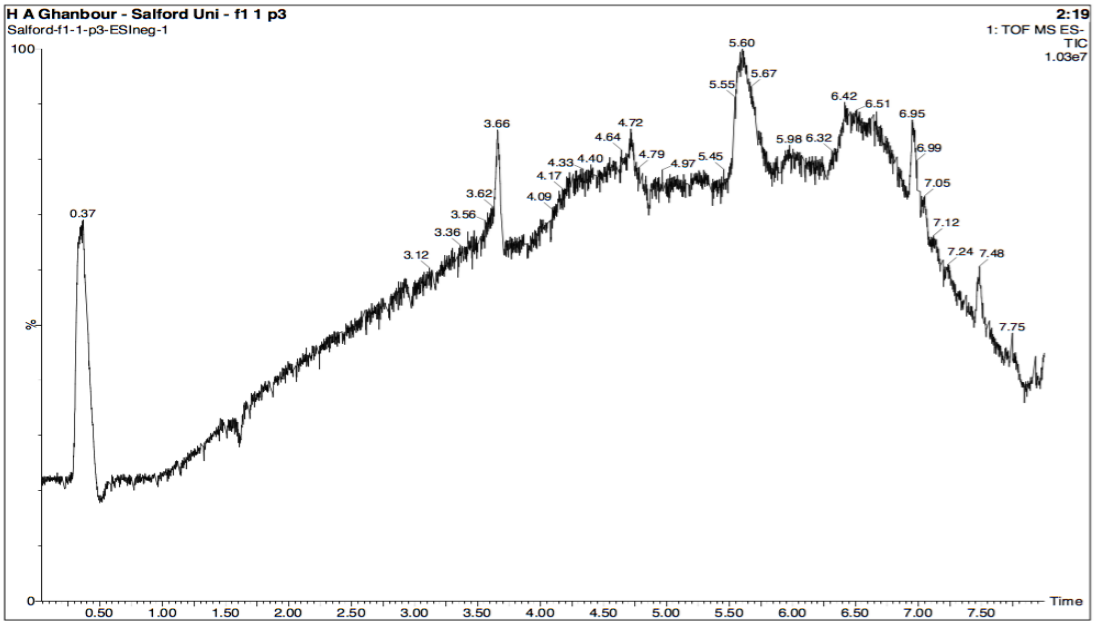
Appendix 3.59: ^{1}H NMR Spectrum of F1 1 P3 in CD_3OD +TMS



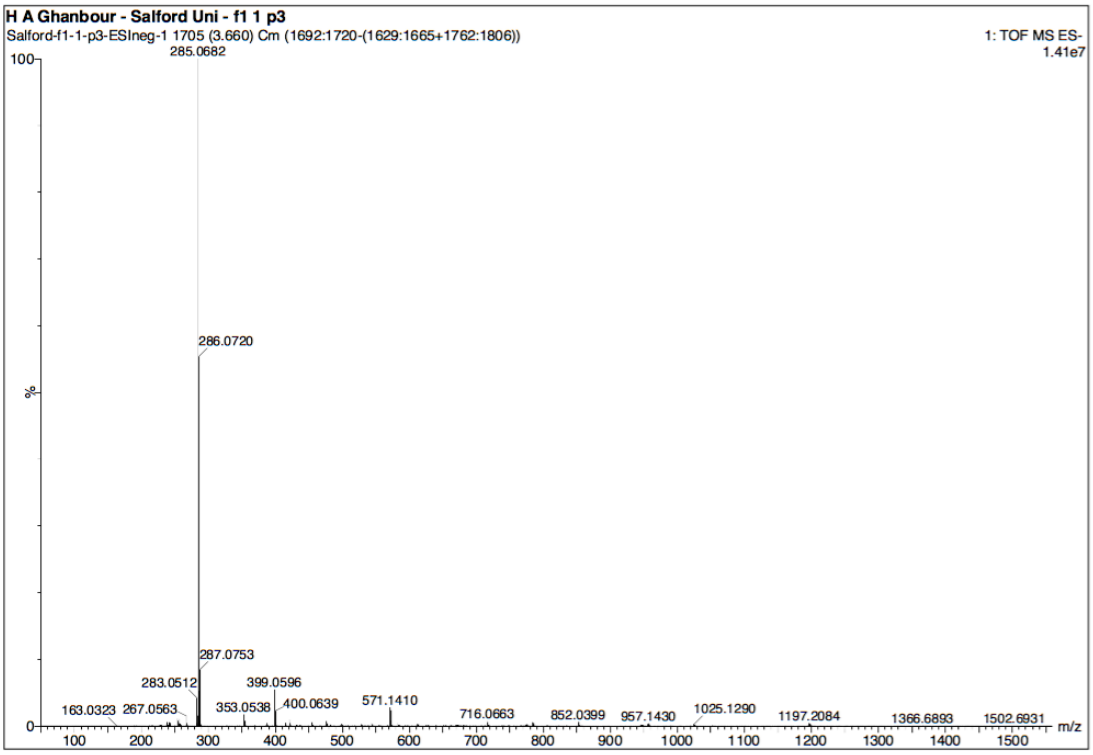
Appendix 3.60: The FTIR spectra (ATR) 4 cm $^{-1}$ (16 Scans) of F1 1 p3



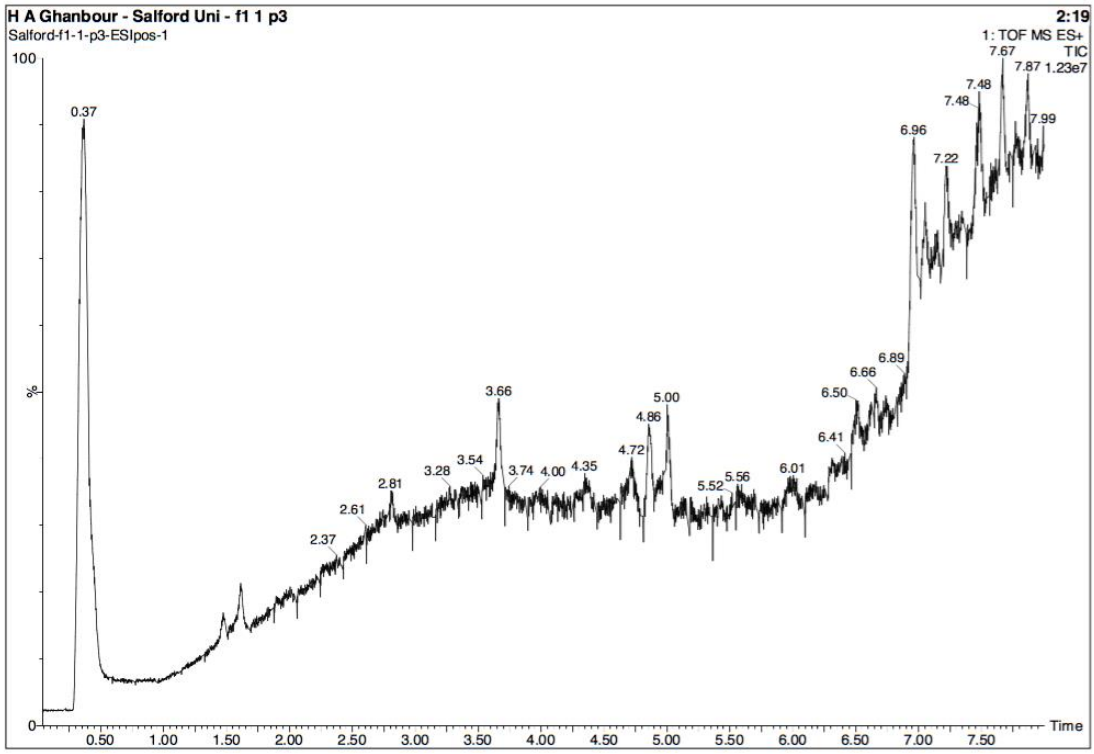
Appendix 3.61: The negative ion chromatogram from LC MS-TOF of F1 1 p3



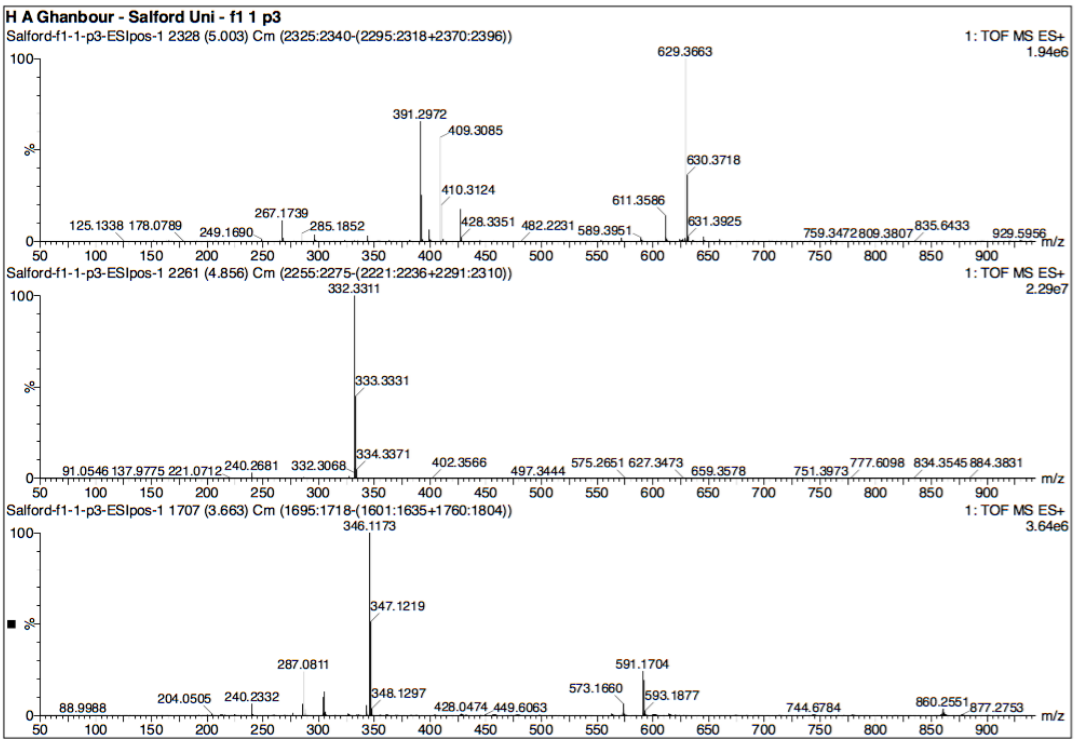
Appendix 3.62: The negative ion spectrum from LC MS-TOF of F1 1 p3



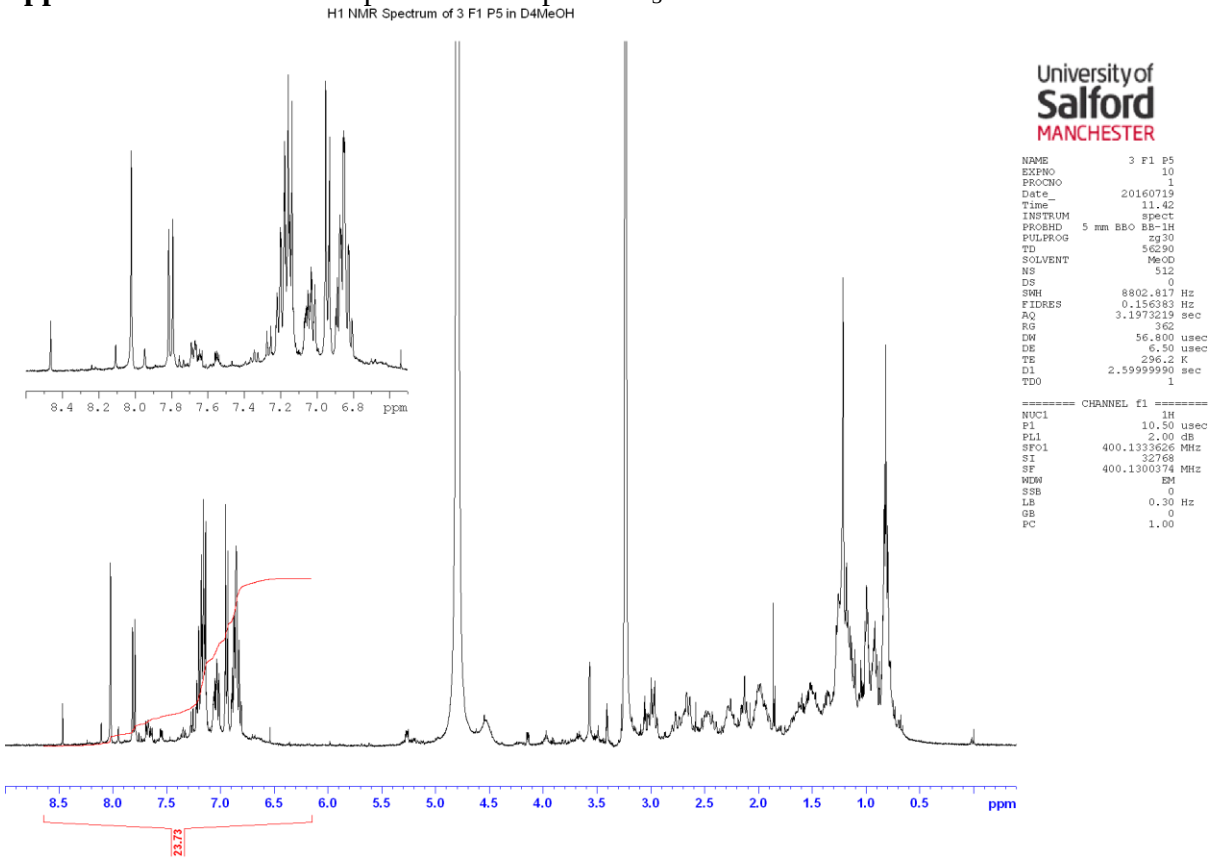
Appendix 3.63: The positive ion chromatogram from LC MS-TOF of F1 1 p3



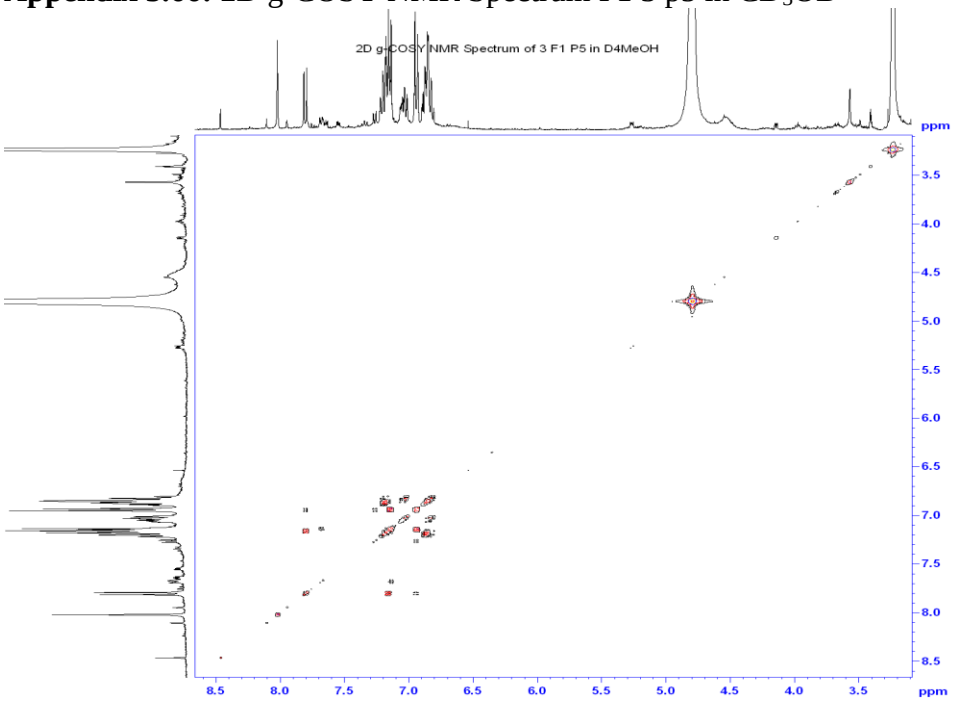
Appendix 3.64: The negative ion spectrum from LC MS-TOF of F1 1 p3



Appendix 3.65: H NMR Spectrum F1 3 p5in CD_3OD+TMS



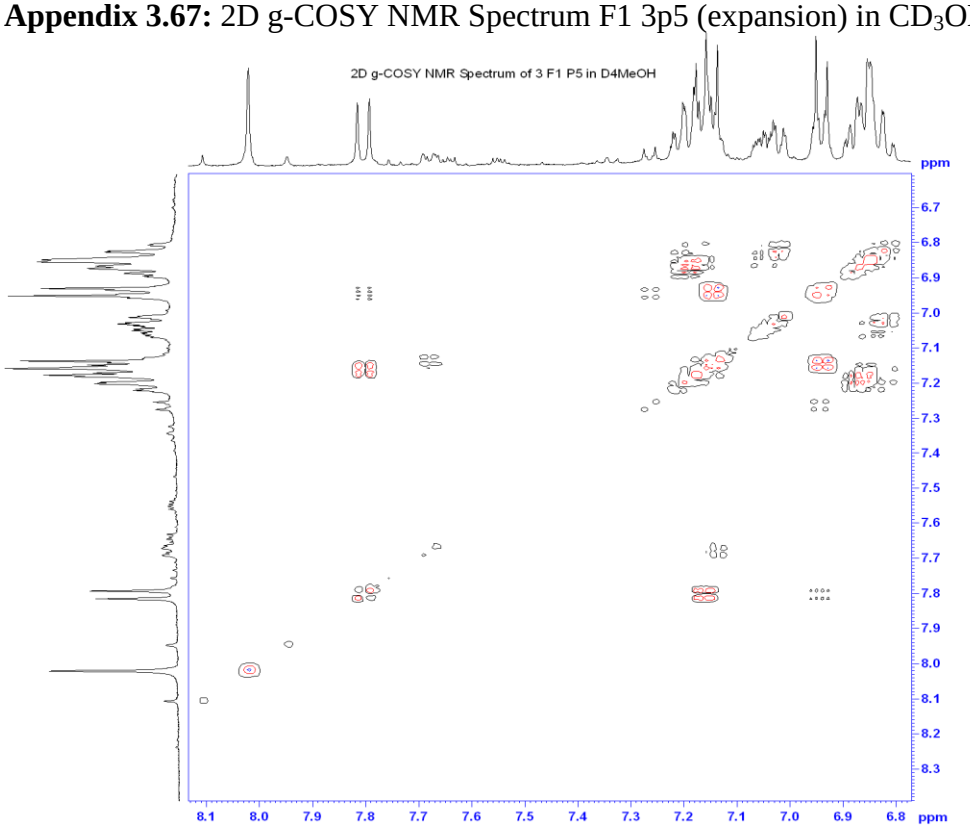
Appendix 3.66: 2D g-COSY NMR Spectrum F1 3 p5 in CD_3OD



University of Salford MANCHESTER

```
NAME                  3 F1 P5
EXPNO                      11
PROCNO                      1
Date_              20160719
Time_                  15.40
INSTRUM                spect
PROBHD   5 mm BBO BB-1H
PULPROG              cosygpqf
TD                       2048
SOLVENT                  MeOD
NS                          8
DS                          8
SWH                2232.143 Hz
FIDRES             1.089913 Hz
AQ                 0.4588020 sec
RG                     161.3
DW                     224.000 usec
DE                        6.50 usec
TE                      296.2 K
D0                 0.00000300 sec
D1                 1.21983194 sec
D13                0.00000400 sec
D16                0.00010000 sec
IN0                0.00044800 sec

======== CHANNEL f1 ========
NUC1                       1H
P0                     11.60 usec
P1                     11.60 usec
PL1                     2.00 dB
SFO1              400.1323879 MHz

====== GRADIENT CHANNEL =====
GPNAM1                SINE.100
GPZ1                     10.00 %
P16                    1000.00 usec
NDO                         1
TD                        128
SFO1              400.1324 MHz
FIDRES             17.438616 Hz
SW                     5.579 ppm
FnMODE                    QF
SI                      1024
SF                400.1300374 MHz
WDW                    SINE
SSB                       0
LB                     0.00 Hz
GB                        0
PC                     1.40
SI                      1024
MC2                       QF
SF                400.1300374 MHz
WDW                    SINE
SSB                       0
LB                     0.00 Hz
GB                        0
```

Appendix 3.67: 2D g-COSY NMR Spectrum F1 3p5 (expansion) in CD_3OD



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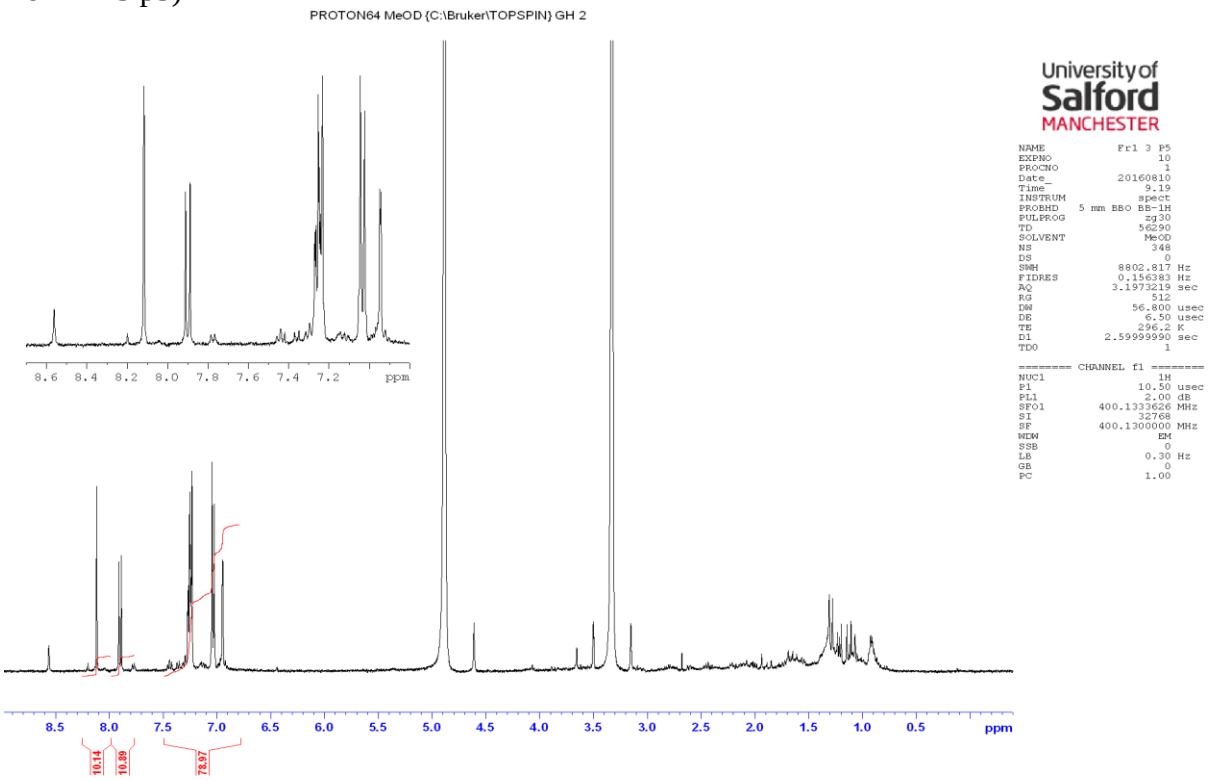
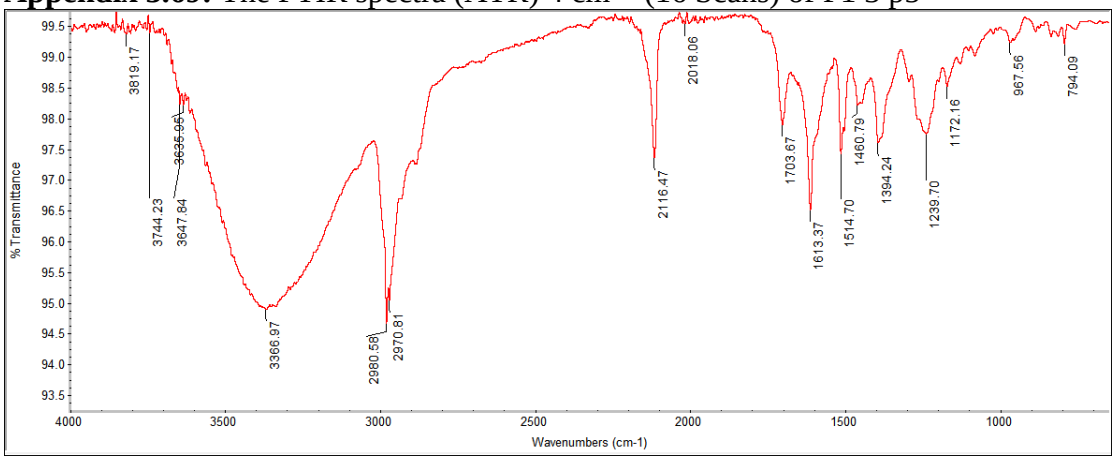
```
NAME                  3 F1 P5
EXPNO                      11
PROCNO                      1
Date_              20160719
Time_                  15.40
INSTRUM                spect
PROBHD   5 mm BBO BB-1H
PULPROG              cosygpqf
TD                       2048
SOLVENT                  MeOD
NS                          8
DS                          8
SWH                2232.143 Hz
FIDRES             1.089913 Hz
AQ                 0.4588020 sec
RG                     161.3
DW                     224.000 usec
DE                        6.50 usec
TE                      296.2 K
D0                 0.00000300 sec
D1                 1.21983194 sec
D13                0.00000400 sec
D16                0.00010000 sec
IN0                0.00044800 sec

======== CHANNEL f1 ========
NUC1                       1H
P0                     11.60 usec
P1                     11.60 usec
PL1                     2.00 dB
SFO1              400.1323879 MHz

====== GRADIENT CHANNEL =====
GPNAM1                SINE.100
GPZ1                     10.00 %
P16                    1000.00 usec
NDO                         1
TD                        128
SFO1              400.1324 MHz
FIDRES             17.438616 Hz
SW                     5.579 ppm
FnMODE                    QF
SI                      1024
SF                400.1300374 MHz
WDW                    SINE
SSB                       0
LB                     0.00 Hz
GB                        0
PC                     1.40
SI                      1024
MC2                       QF
SF                400.1300374 MHz
WDW                    SINE
SSB                       0
LB                     0.00 Hz
GB                        0
```

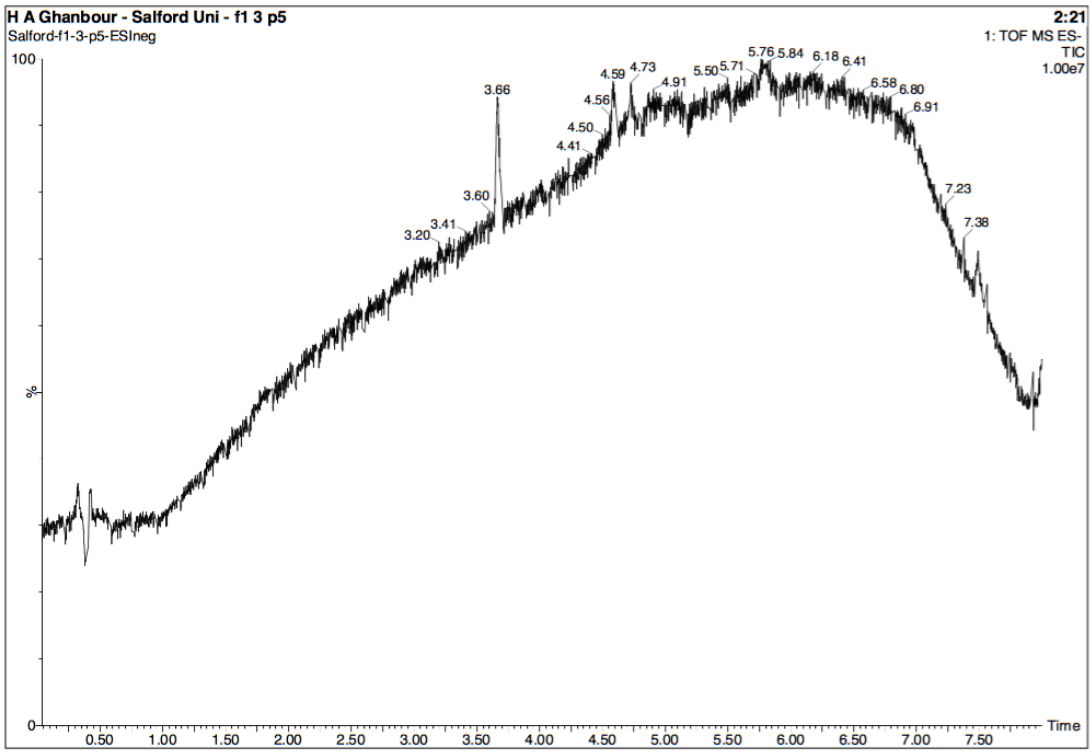
Appendix 3.68: ^{1}H NMR Spectrum of F1 3 p5 in CD_3OD+TMS (Purified compound from F1 3 p5)



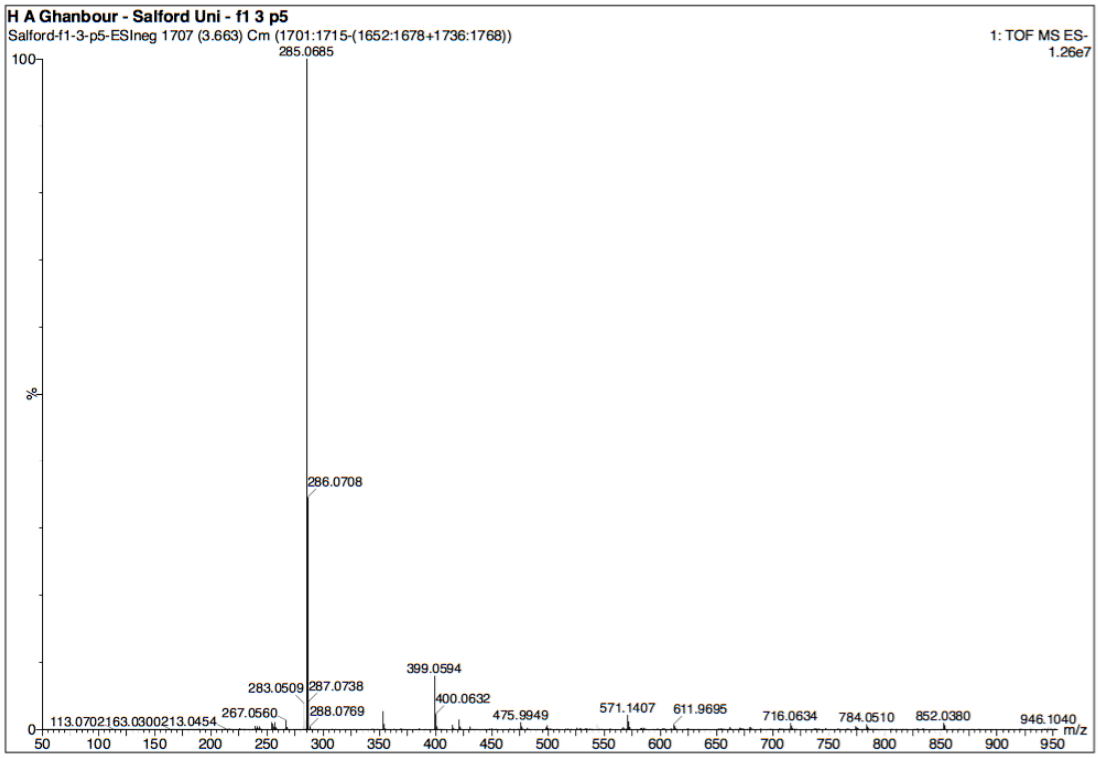
Appendix 3.69: The FTIR spectra (ATR) 4 cm $^{-1}$ (16 Scans) of F1 3 p5



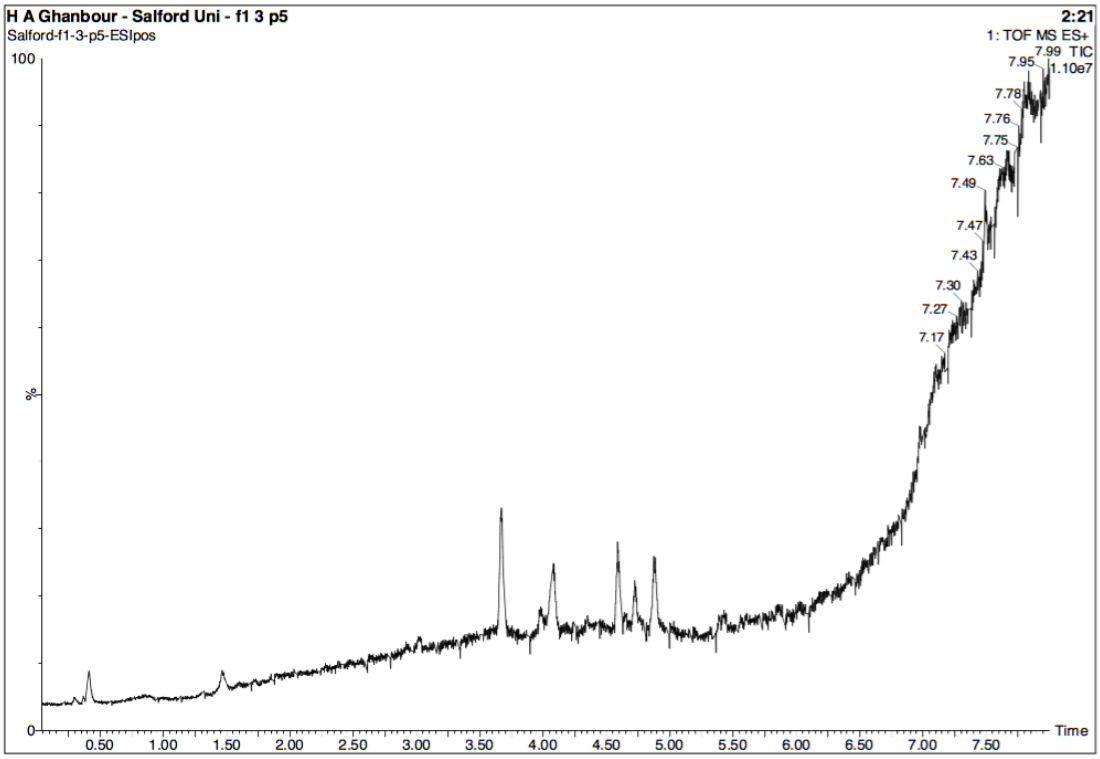
Appendix 3.70: The negative ion chromatogram from LC MS-TOF of F1 3 p5



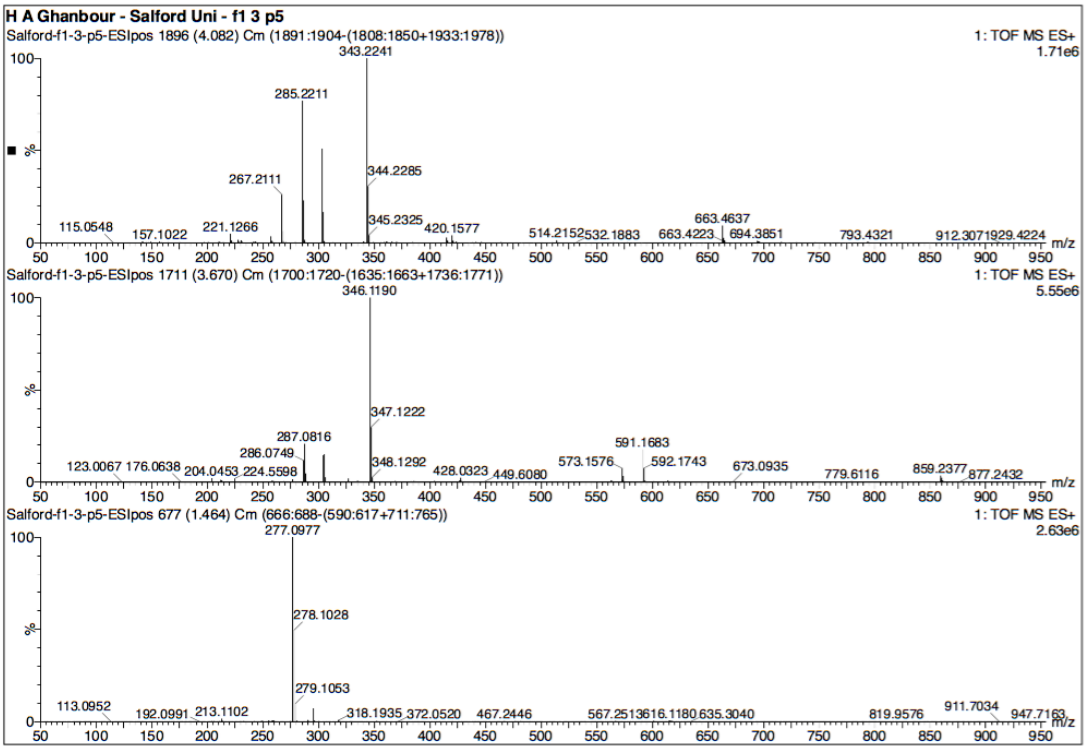
Appendix 3.71: The negative ion spectrum from LC MS-TOF of F1 3 p5



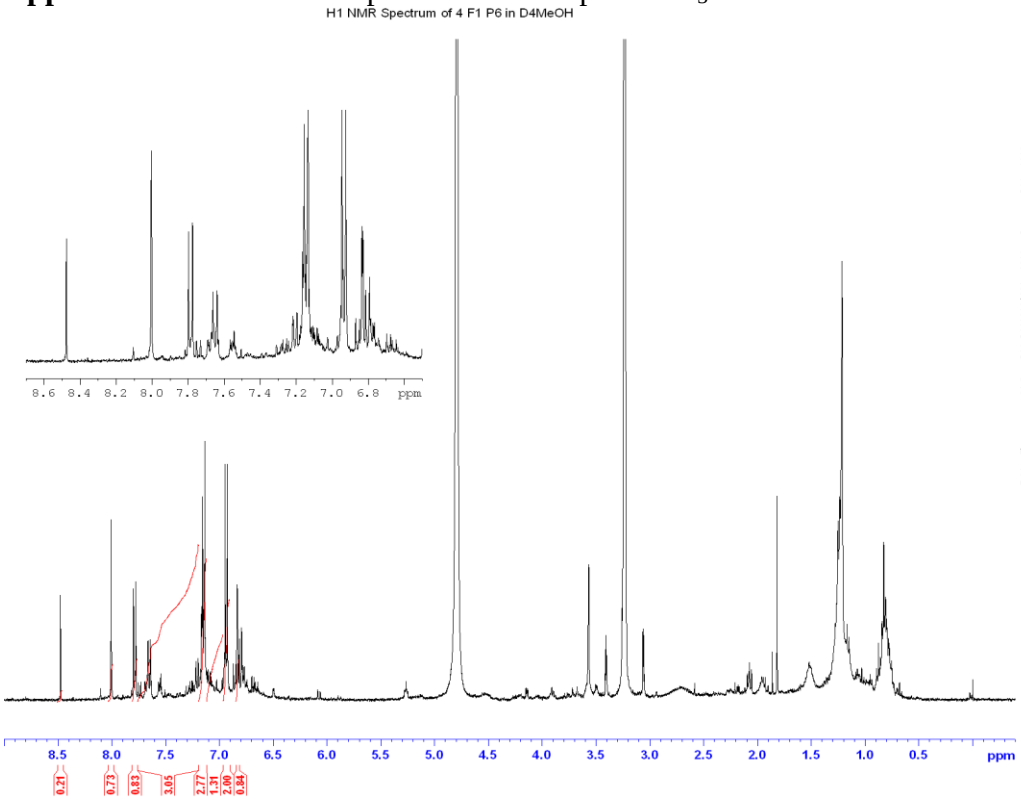
Appendix 3.72: The positive ion chromatogram from LC MS-TOF of F1 3 p5



Appendix 3.73: The positive ion spectrum from LC MS-TOF of F1 3 p5



Appendix 3.74: H NMR Spectrum of F1 4 p6 in CD_3OD+TMS



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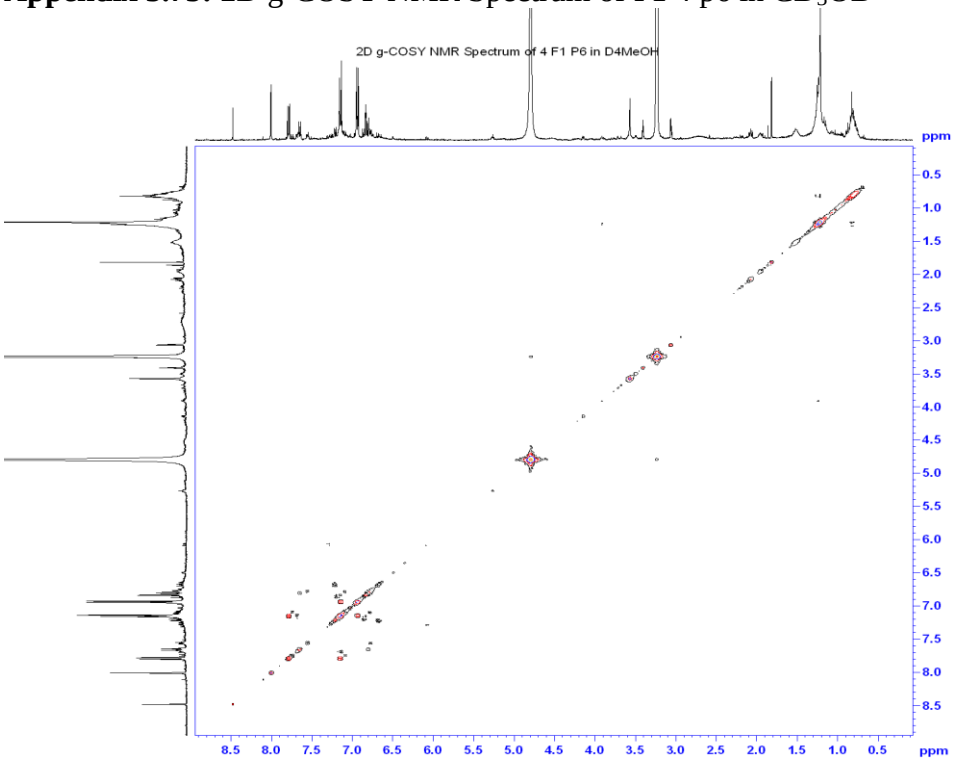
```
NAME                 4 F1 P6
EXPNO                   10
PROCNO                   1
Date_             20160718
Time                 10.36
INSTRUM              spect
PROBHD     5 mm BBO BB-1H
PULPROG              zg30
TD                  56290
SOLVENT              MeOD
NS                     256
DS                       0
SWH               8802.817 Hz
FIDRES            0.156383 Hz
AQ                3.1973219 sec
RG                     512
DW                  56.800 usec
DE                   6.50 usec
TE                  296.2 K
D1                2.59999990 sec
TD0                      1

======== CHANNEL f1 ========
NUC1                    1H
P1                   10.50 usec
PL1                   2.00 dB
SFO1          400.13336266 MHz
SI                   32768
SF            400.1300375 MHz
WDW                     EM
SSB                      0
LB                    0.30 Hz
GB                       0
PC                    1.00
```

Appendix 3.75: 2D g-COSY NMR Spectrum of F1 4 p6 in CD_3OD



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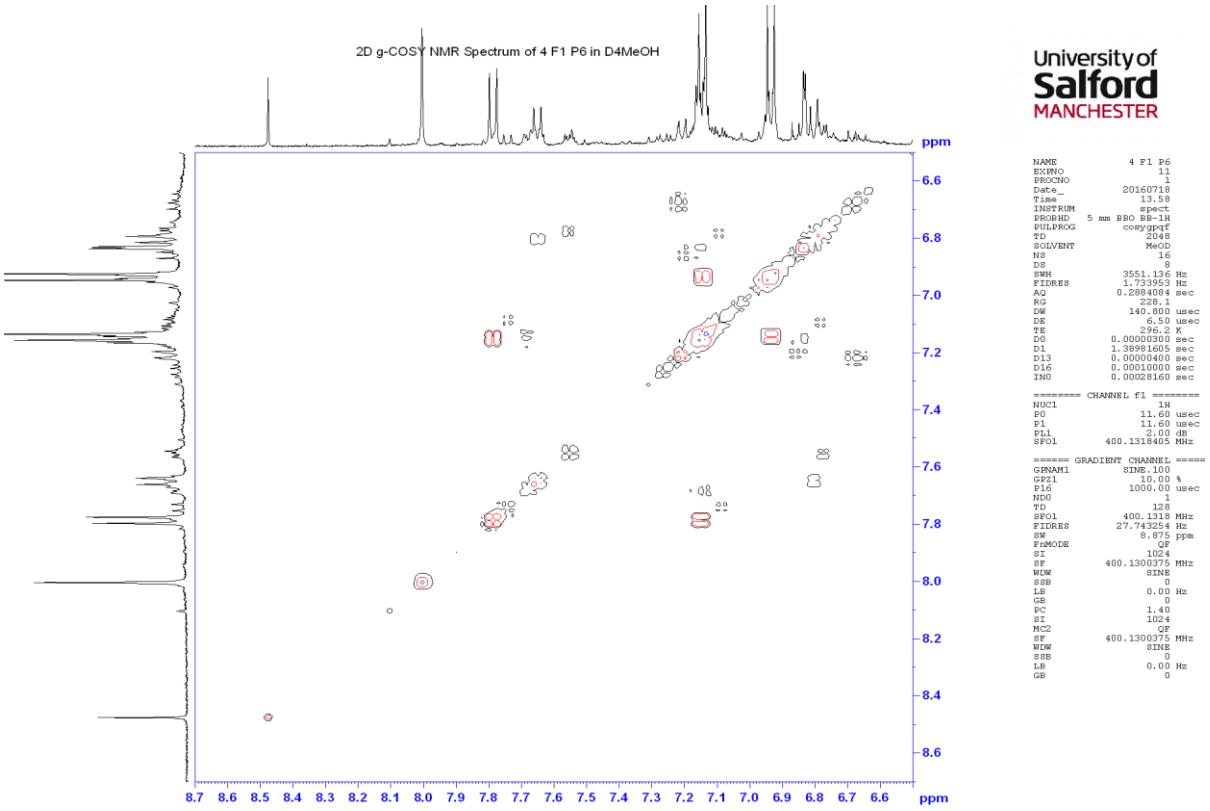
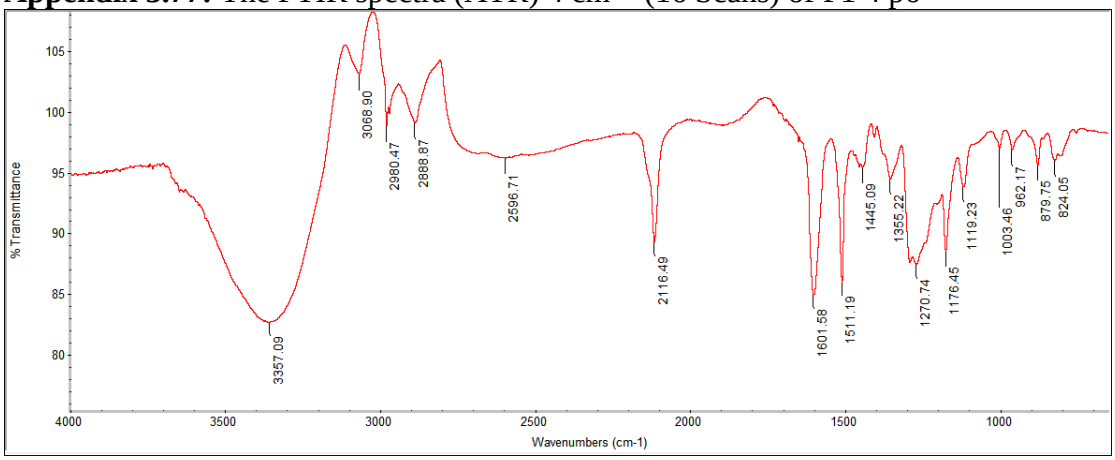
```
NAME                 4 F1 P6
EXPNO                   11
PROCNO                   1
Date_             20160718
Time                 13.58
INSTRUM              spect
PROBHD     5 mm BBO BB-1H
PULPROG           cosygpqf
TD                    2048
SOLVENT              MeOD
NS                      16
DS                       8
SWH               3551.136 Hz
FIDRES            1.733953 Hz
AQ                0.2884084 sec
RG                   228.1
DW                  140.800 usec
DE                   6.50 usec
TE                  296.2 K
D0                0.00000300 sec
D1                1.38981605 sec
D13               0.00000400 sec
D16               0.00010000 sec
IN0               0.00028160 sec

======== CHANNEL f1 ========
NUC1                    1H
P0                   11.60 usec
P1                   11.60 usec
PL1                   2.00 dB
SFO1          400.1319405 MHz

====== GRADIENT CHANNEL =====
GPNAM1             SINE.100
GPZ1                 10.00 %
P16                1000.00 usec
ND0                      1
TD                     128
SFO1          400.1318 MHz
FIDRES          27.743254 Hz
SW                 8.875 ppm
FnMODE                  QF
SI                    1024
SF            400.1300375 MHz
WDW                   SINE
SSB                      0
LB                    0.00 Hz
GB                       0
PC                    1.40
SI                    1024
MC2                     QF
SF            400.1300375 MHz
WDW                   SINE
SSB                      0
LB                    0.00 Hz
GB                       0
```

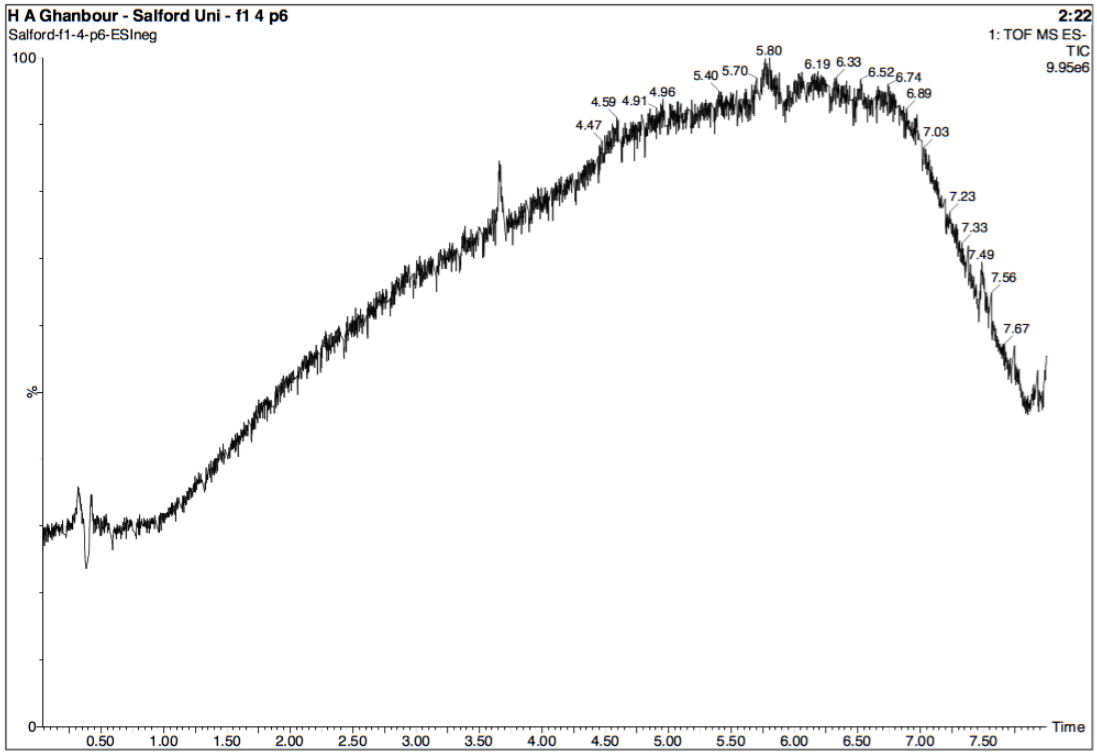
Appendix 3.76: 2D g-COSY NMR Spectrum of F1 4 p6 (expansion) in CD_3OD



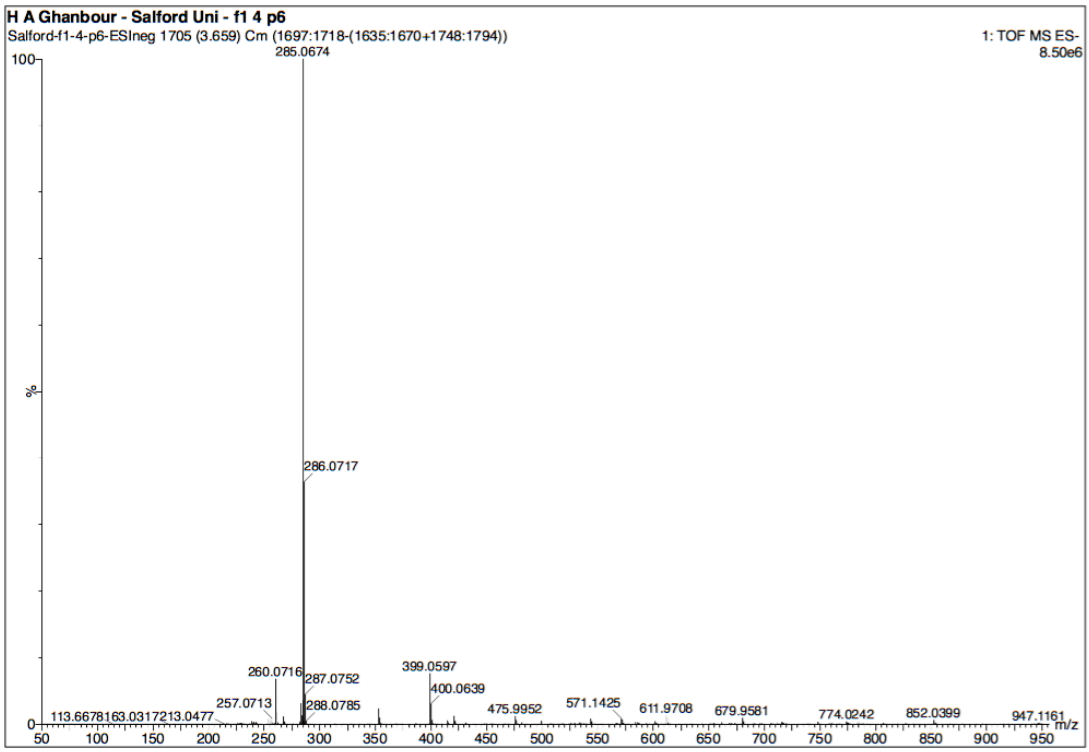
Appendix 3.77: The FTIR spectra (ATR) 4 cm $^{-1}$ (16 Scans) of F1 4 p6



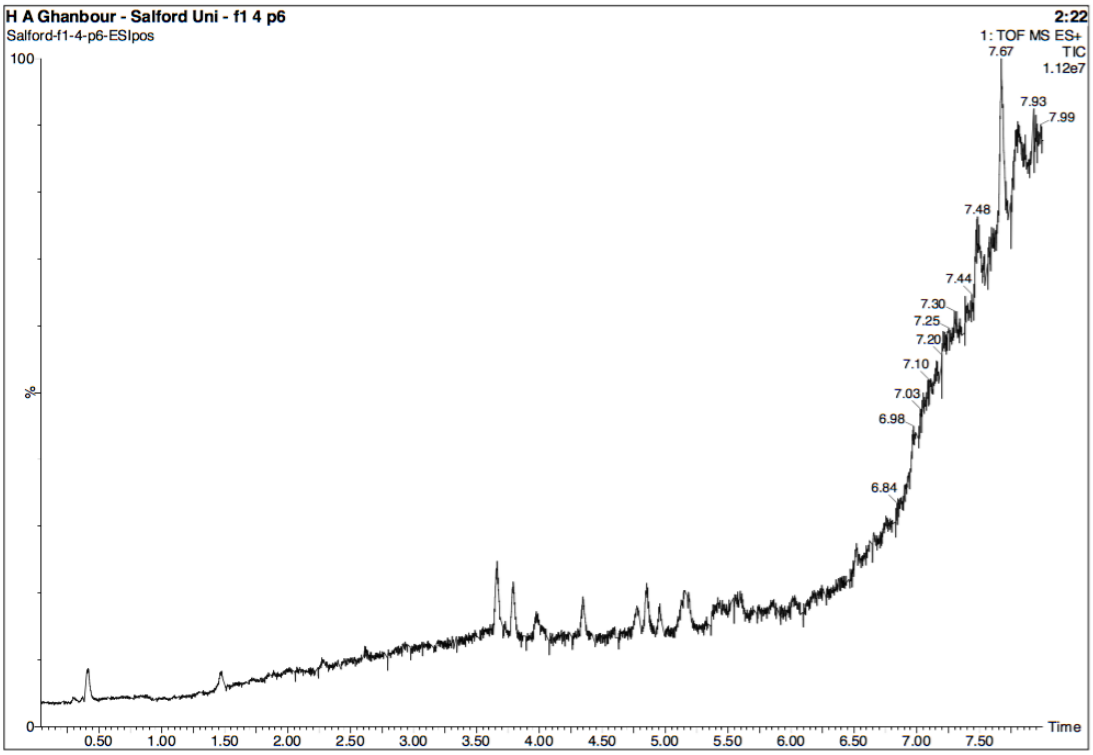
Appendix 3.78: The negative ion chromatogram from LC MS-TOF of F1 4 p6



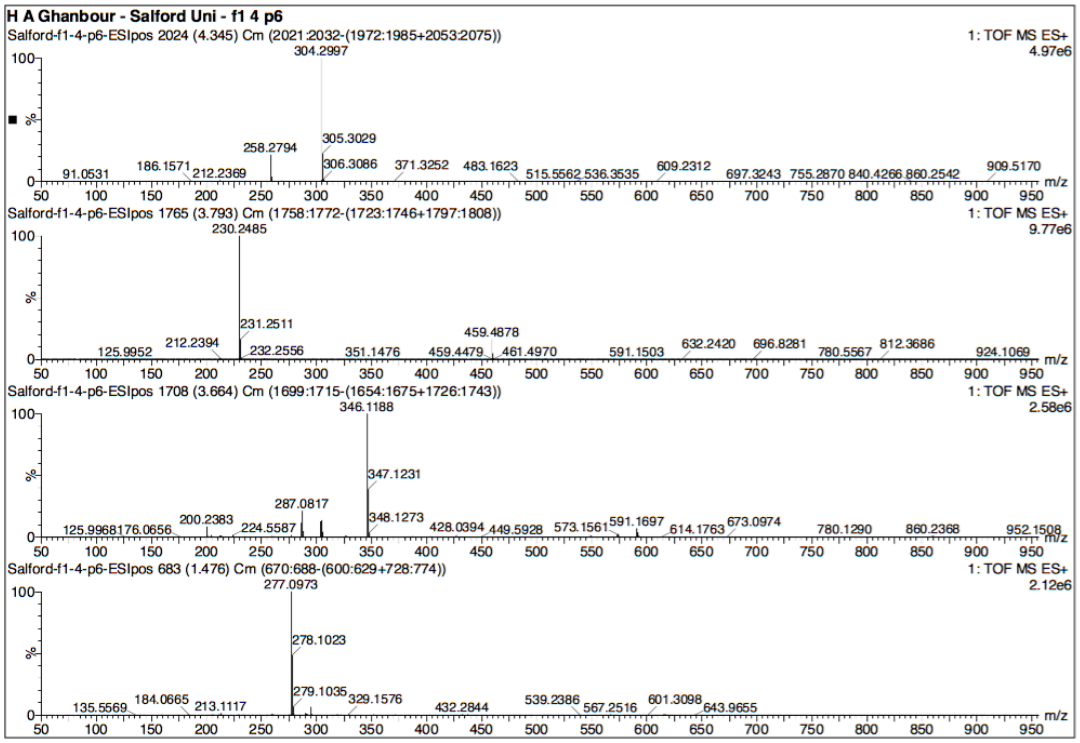
Appendix 3.79: The negative ion spectrum from LC MS-TOF of F1 4 p6



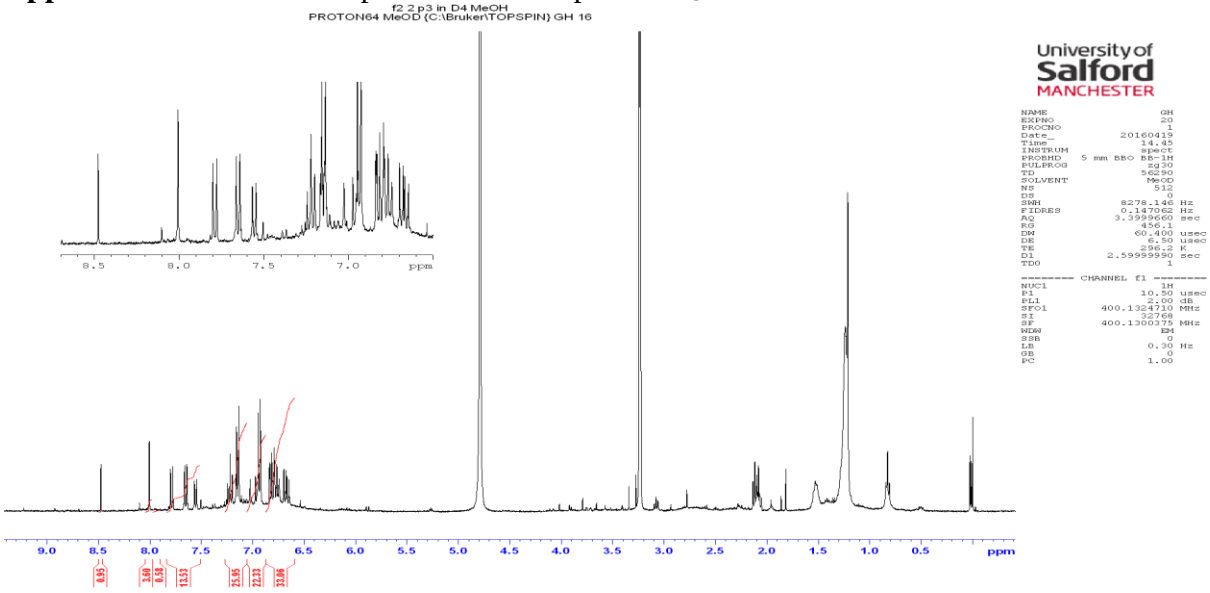
Appendix 3.80: The positive ion chromatogram from LC MS-TOF of F1 4 p6



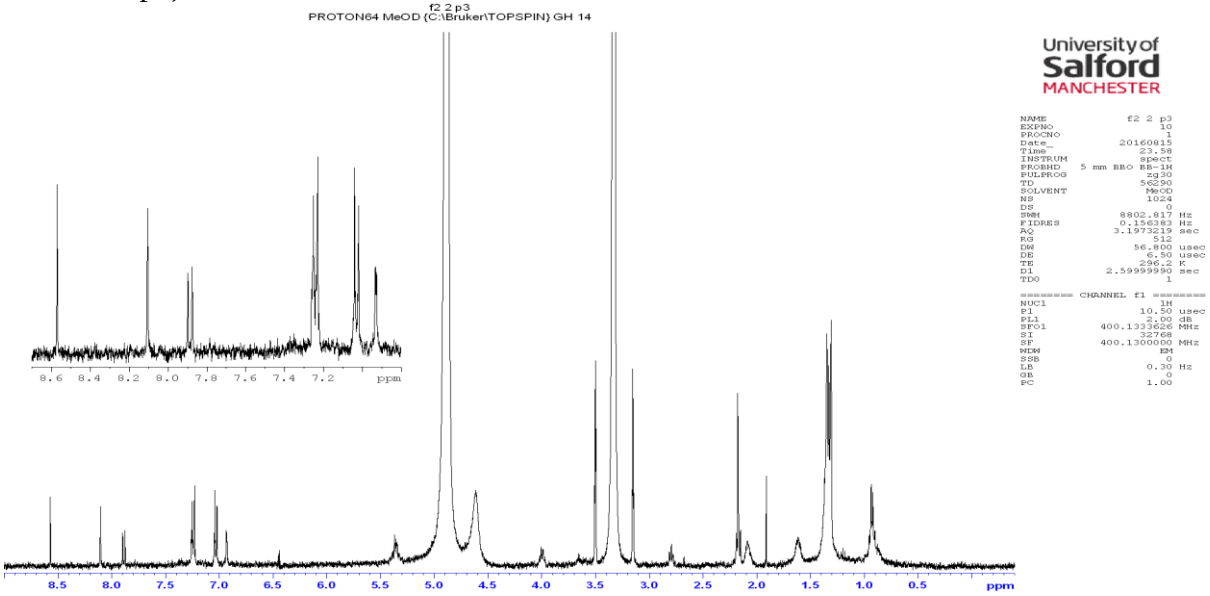
Appendix 3.81: The positive ion spectrum from LC MS-TOF of F1 4 p6



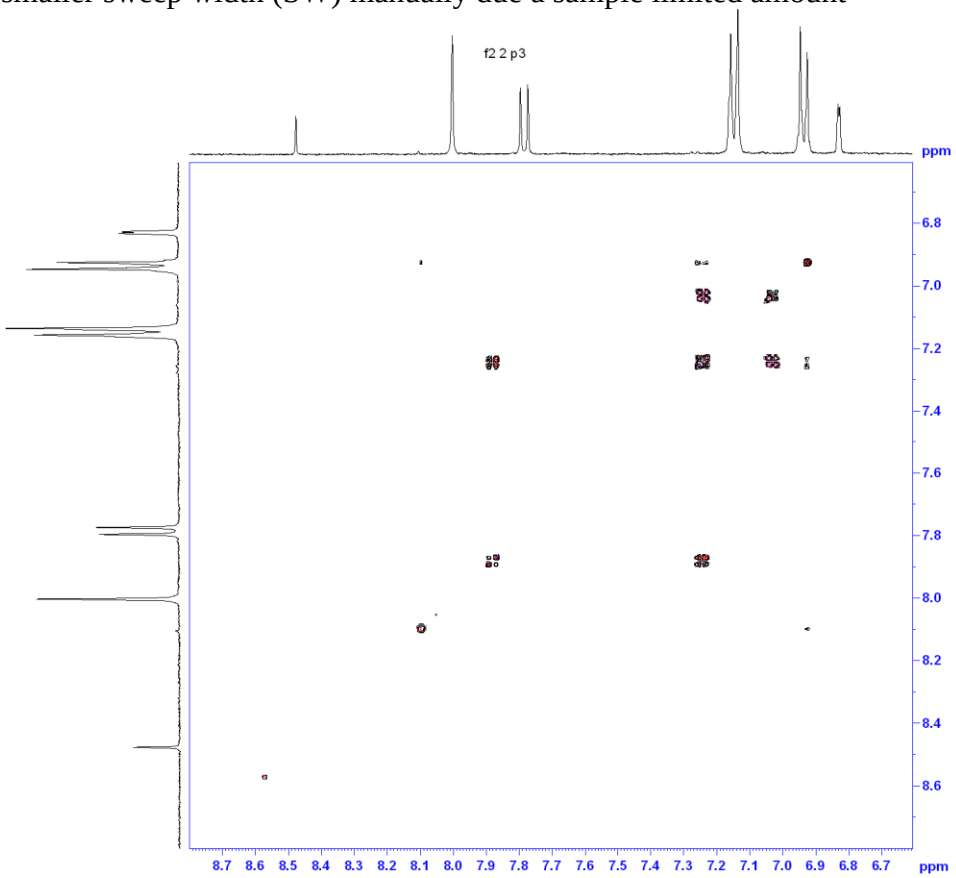
Appendix 3.82: ^{1}H NMR Spectrum of F2 2 p3 in CD_3OD+TMS



Appendix 3.83: H NMR Spectrum of F2 2 p3 in CD_3OD+TMS (Purified compound from F2 2 p3)



Appendix 3.84: 2D g-COSY NMR Spectrum F2 2 p3 in CD_3OD acquired with smaller sweep width (SW) manually due a sample limited amount



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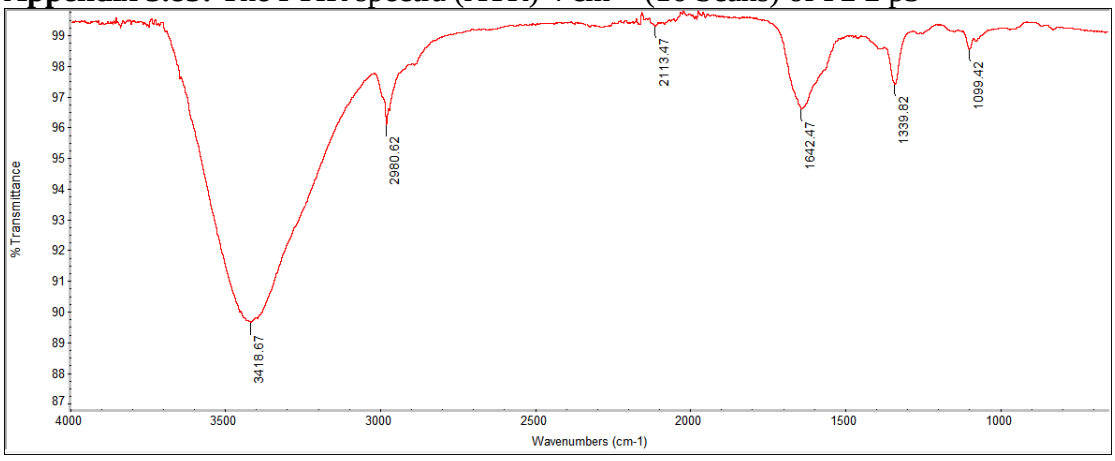
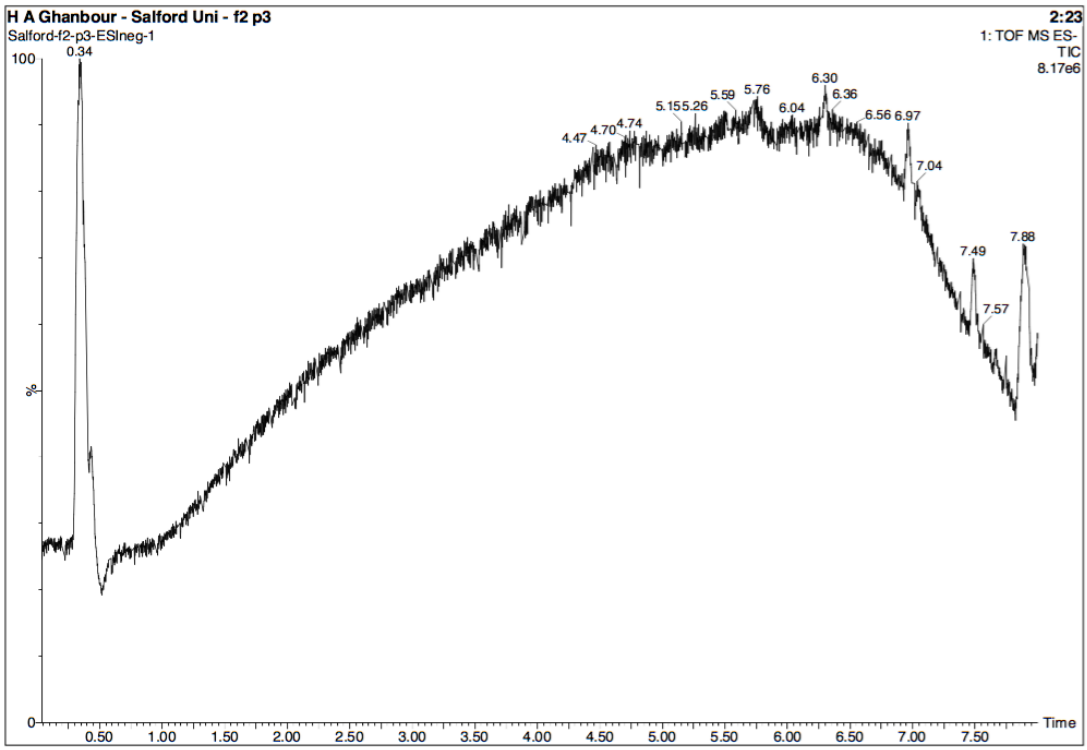
EXPNO	30
PROCNO	1
Date_	20160804
Time	15.00
INSTRUM	spect
PROBHD	5 mm BBO BB-1H
PULPROG	cosygpqf
TD	2048
SOLVENT	MeOD
NS	8
DS	8
SWH	877.809 Hz
FIDRES	0.428618 Hz
AQ	1.1665908 sec
RG	4096
DW	569.600 usec
DE	6.50 usec
TE	296.2 K
D0	0.00000300 sec
D1	1.33452106 sec
D13	0.00000400 sec
D16	0.00010000 sec
IN0	0.00113920 sec
======== CHANNEL f1 ========	
NUC1	1H
P0	11.60 usec
P1	11.60 usec
PL1	2.00 dB
SFO1	400.1330822 MHz
====== GRADIENT CHANNEL =====	
GPNAM1	SINE.100
GP21	10.00 %
P16	1000.00 usec
ND0	1
TD	128
SFO1	400.1331 MHz
FIDRES	6.857899 Hz
SW	2.194 ppm
FnMODE	QF
SI	1024
SF	400.1300000 MHz
WDW	SINE
SSB	0
LB	0.00 Hz
GB	0
PC	1.40
SI	1024
MC2	QF
SF	400.1300000 MHz
WDW	SINE
SSB	0
LB	0.00 Hz
GB	0

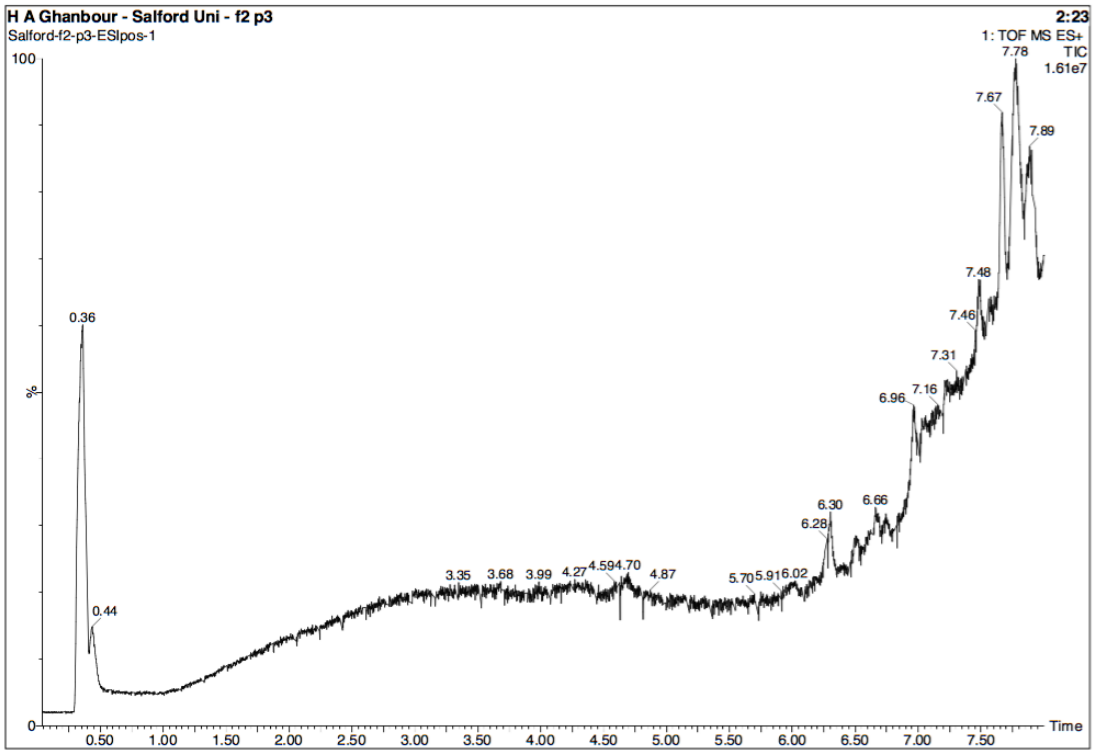
Appendix 3.85: The FTIR spectra (ATR) 4 cm $^{-1}$ (16 Scans) of F2 2 p3



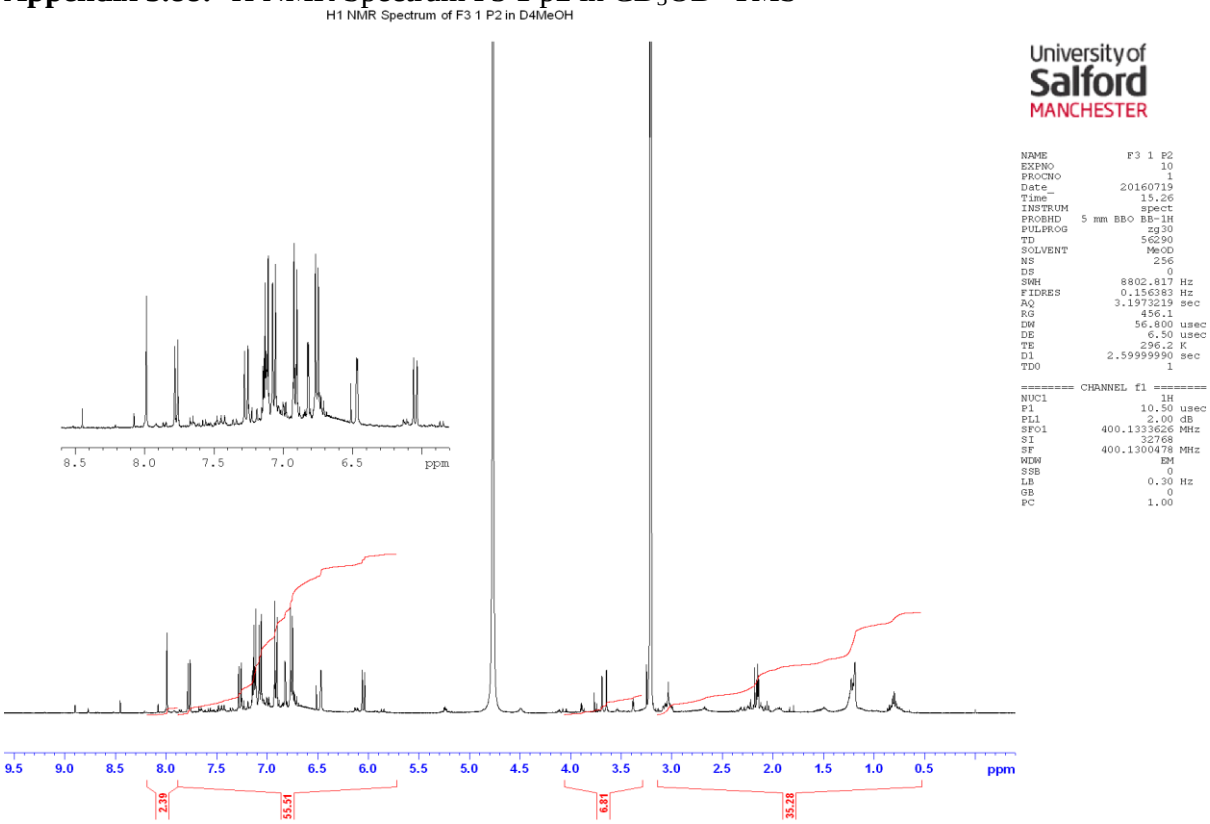
Appendix 3.86: The negative ion chromatogram from LC MS-TOF of F2 p3



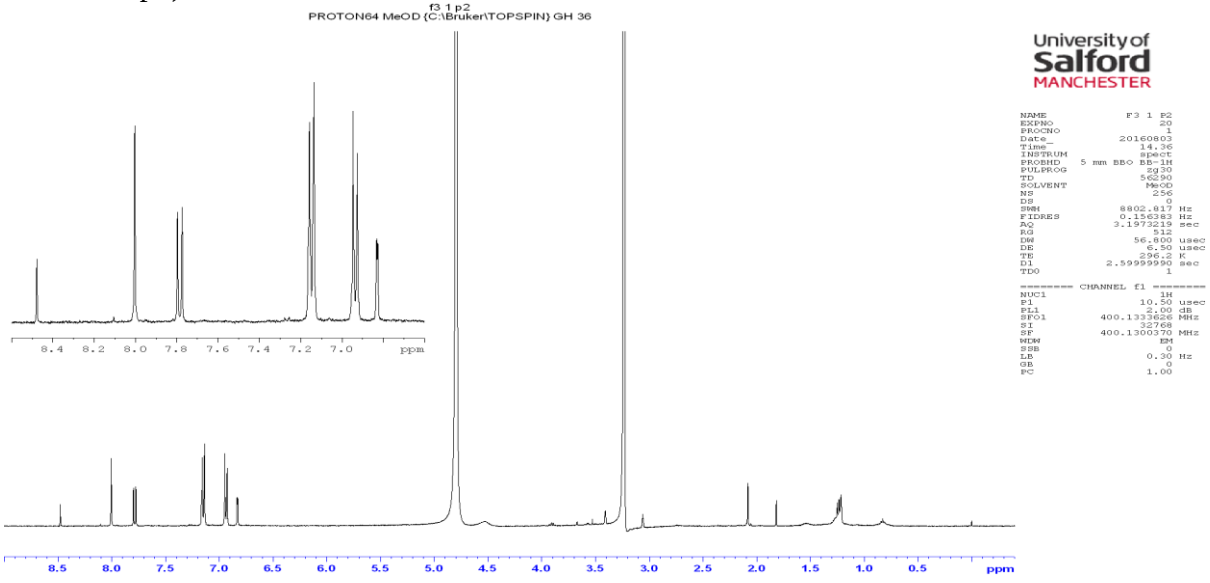
Appendix 3.87: The negative ion chromatogram from LC MS-TOF of F2 p3



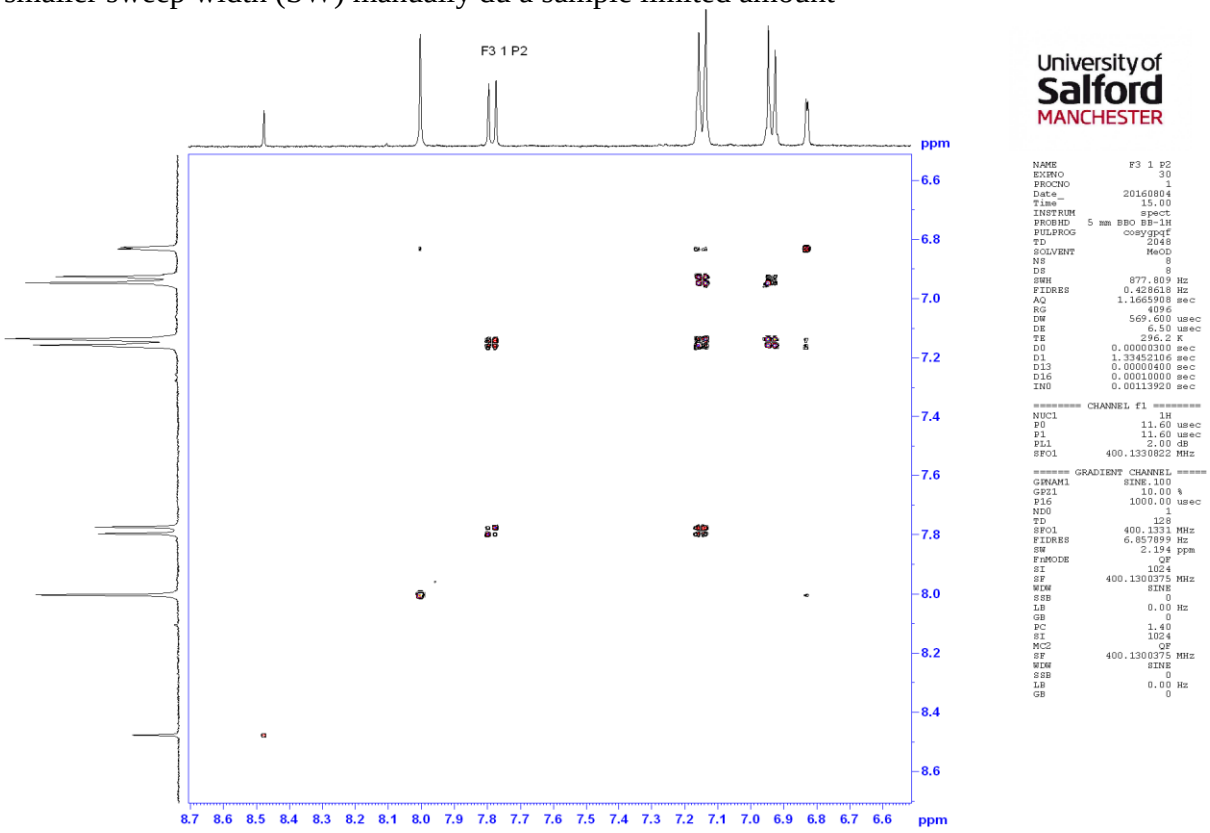
Appendix 3.88: ^{1}H NMR Spectrum F3 1 p2 in CD_3OD+TMS



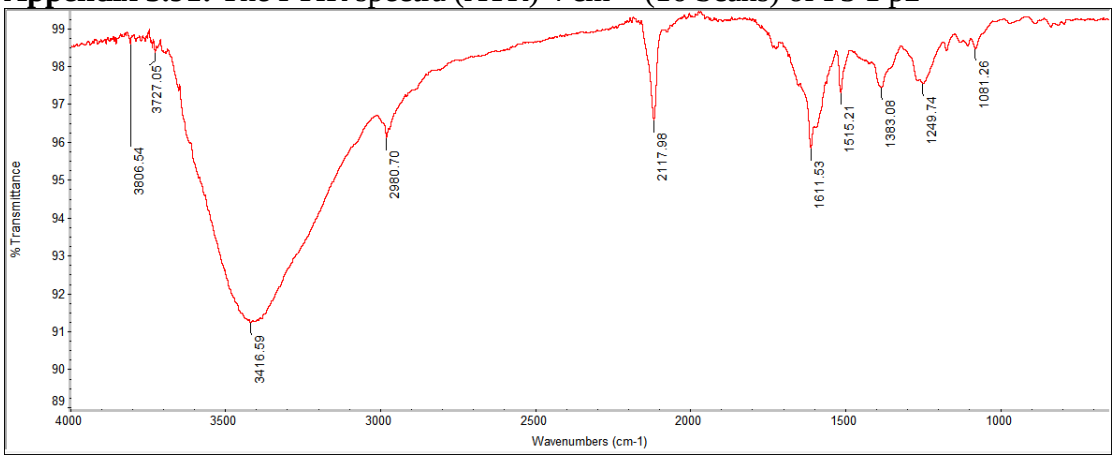
Appendix 3.89: ^{1}H NMR Spectrum of F3 1 p2 in CD_3OD+TMS (Purified compound from F3 1 p2)



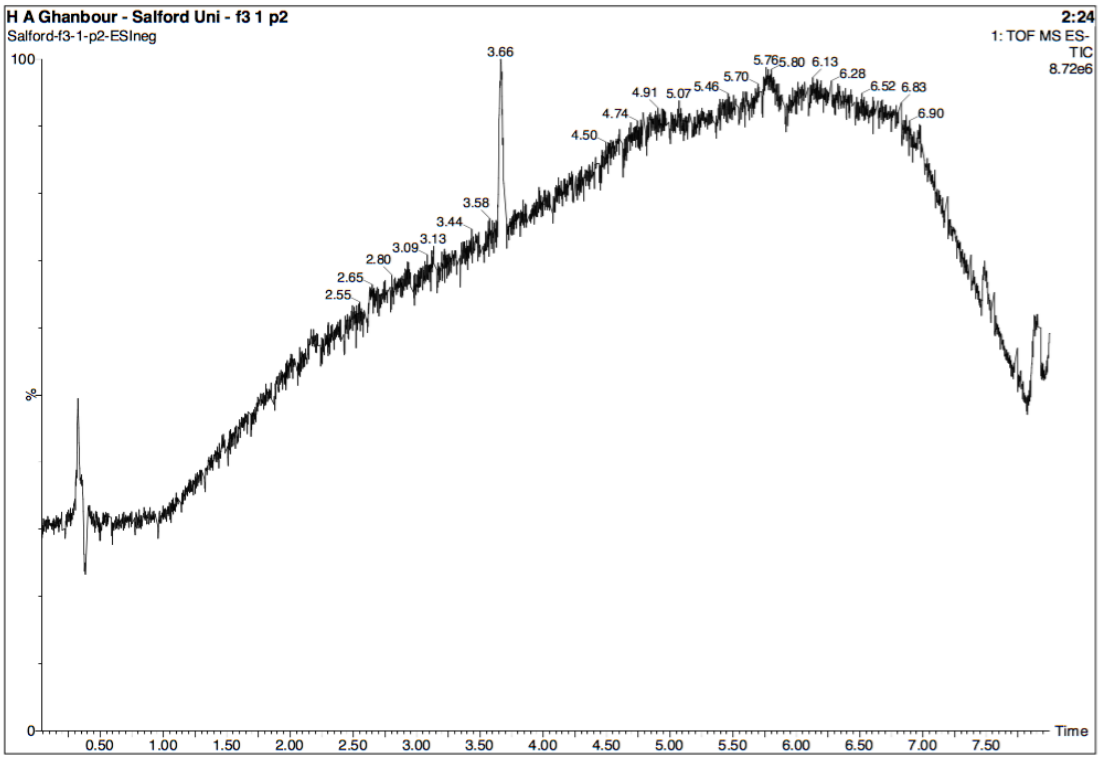
Appendix 3.90: 2D g-COSY NMR Spectrum F3 1 p2 in CD_3OD acquired with smaller sweep width (SW) manually du a sample limited amount



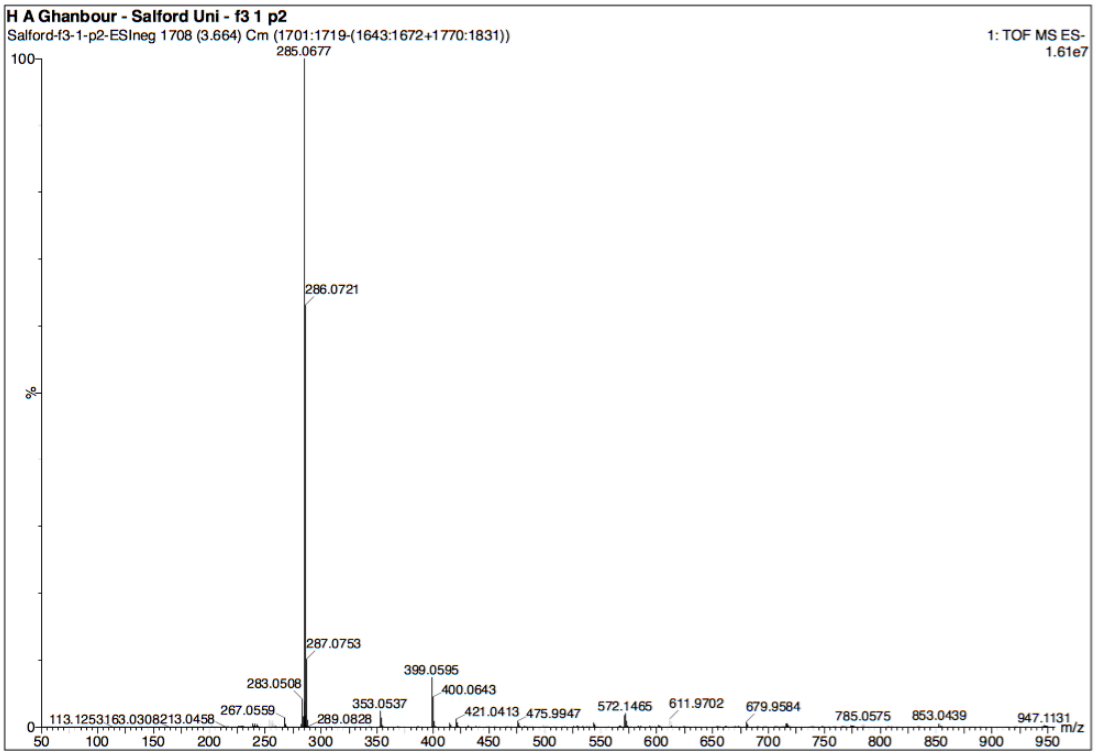
Appendix 3.91: The FTIR spectra (ATR) 4 cm $^{-1}$ (16 Scans) of F3 1 p2



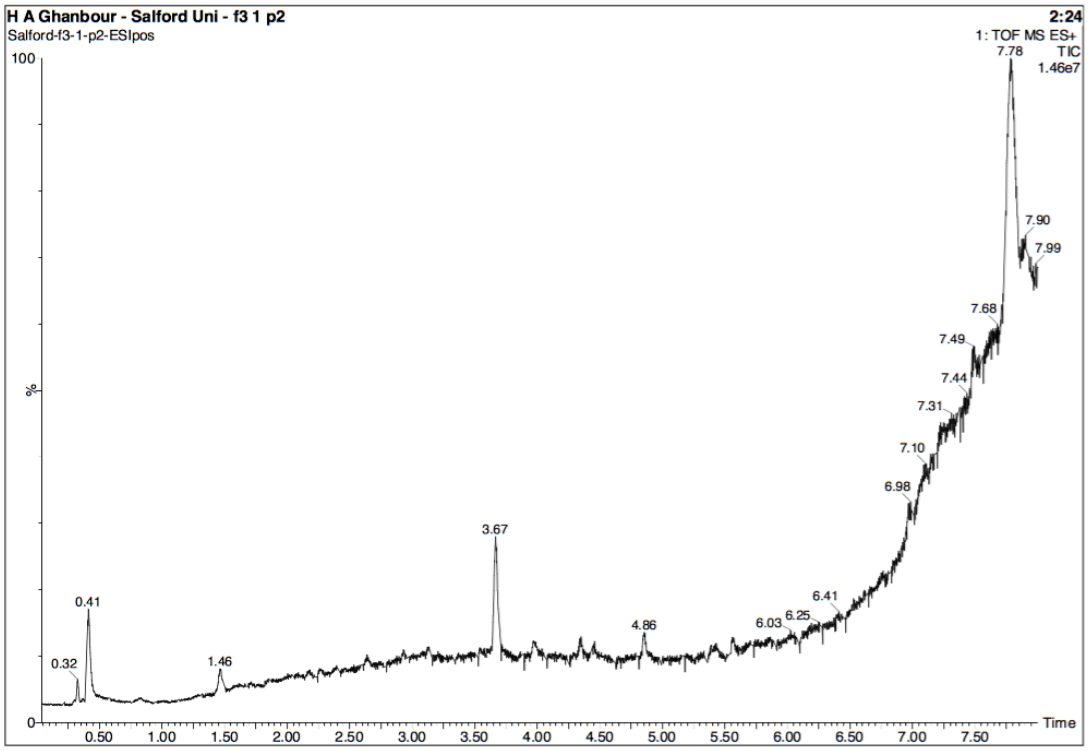
Appendix 3.92: The negative ion chromatogram from LC MS-TOF of F3 1 p2



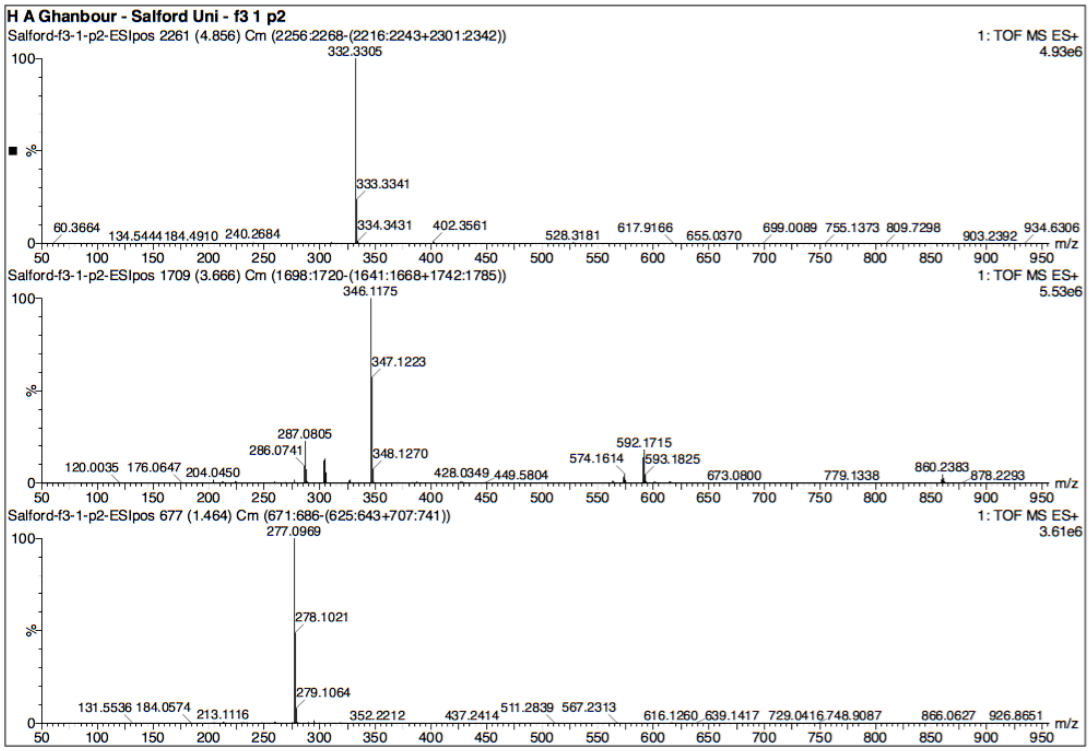
Appendix 3.93: The negative ion spectrum from LC MS-TOF of F3 1 p2



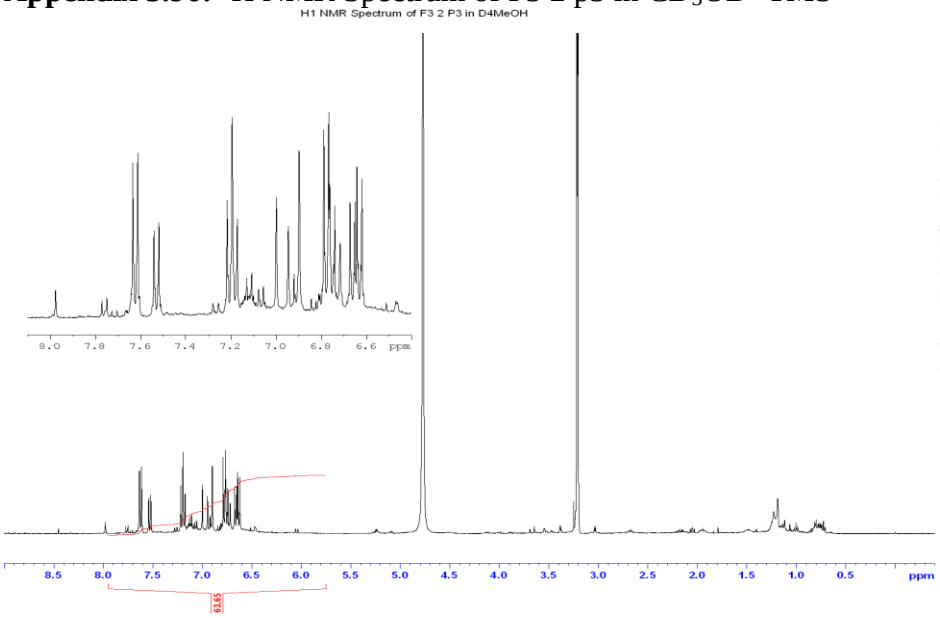
Appendix 3.94: The positive ion chromatogram from LC MS-TOF of F3 1 p2



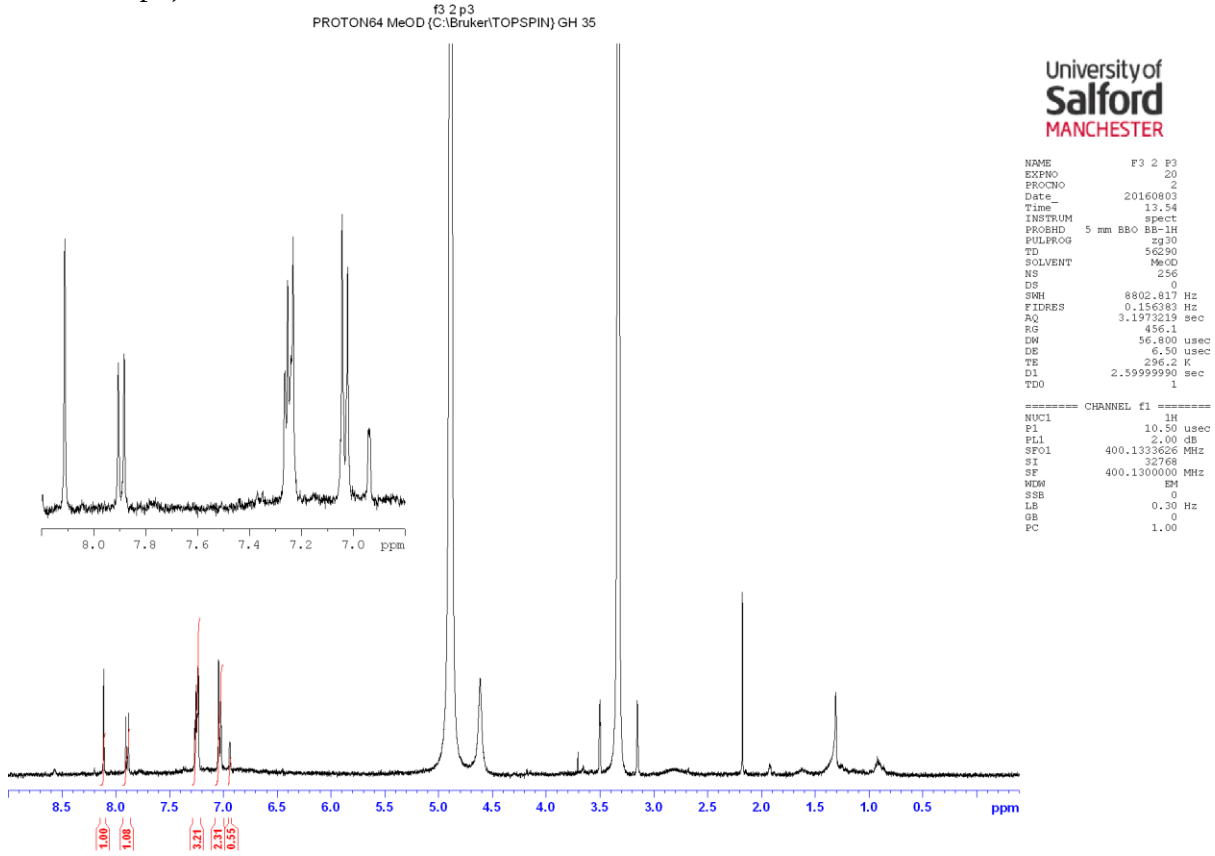
Appendix 3.95: The positive ion spectrum from LC MS-TOF of F3 1 p2



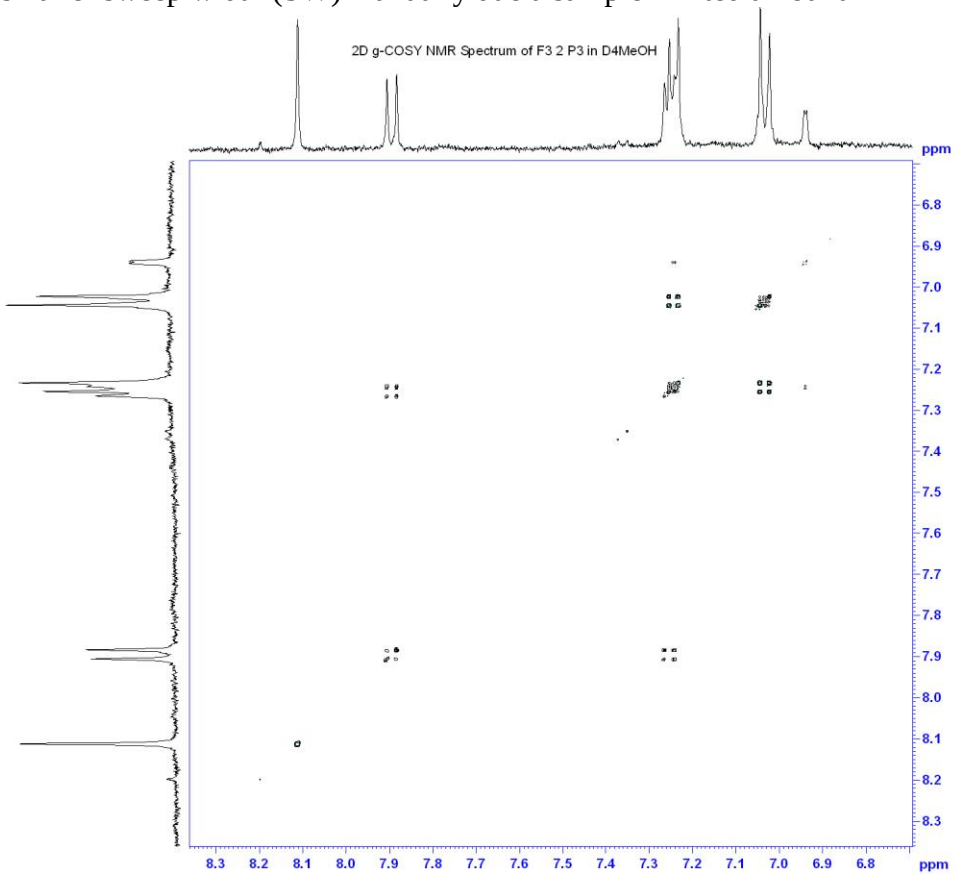
Appendix 3.96: ^{1}H NMR Spectrum of F3 2 p3 in CD_3OD+TMS



Appendix 3.97: ^{1}H NMR Spectrum of F3 2 p3 in CD_3OD+TMS (Purified compound from F3 2 p3)



Appendix 3.98: 2D g-COSY NMR Spectrum of F3 2 p3in CD_3OD acquired with smaller sweep width (SW) manually due a sample limited amount



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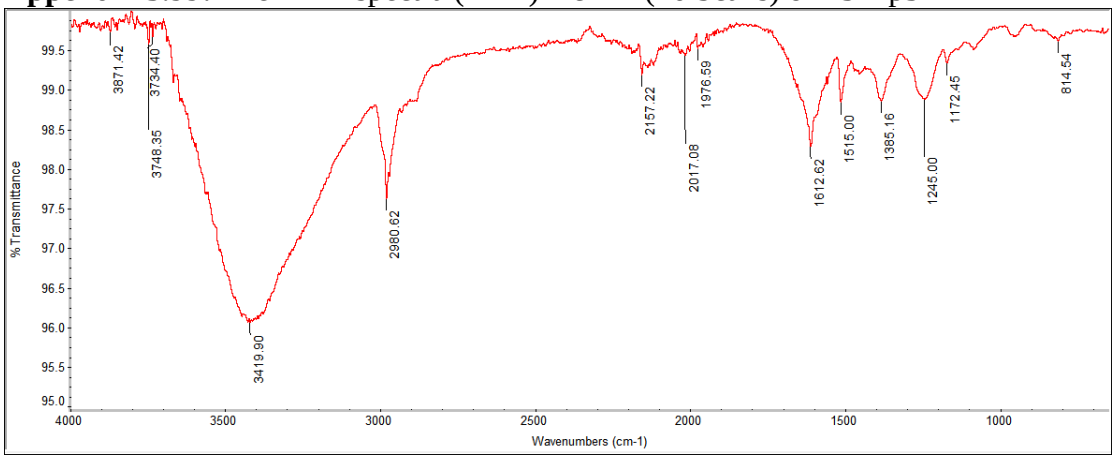
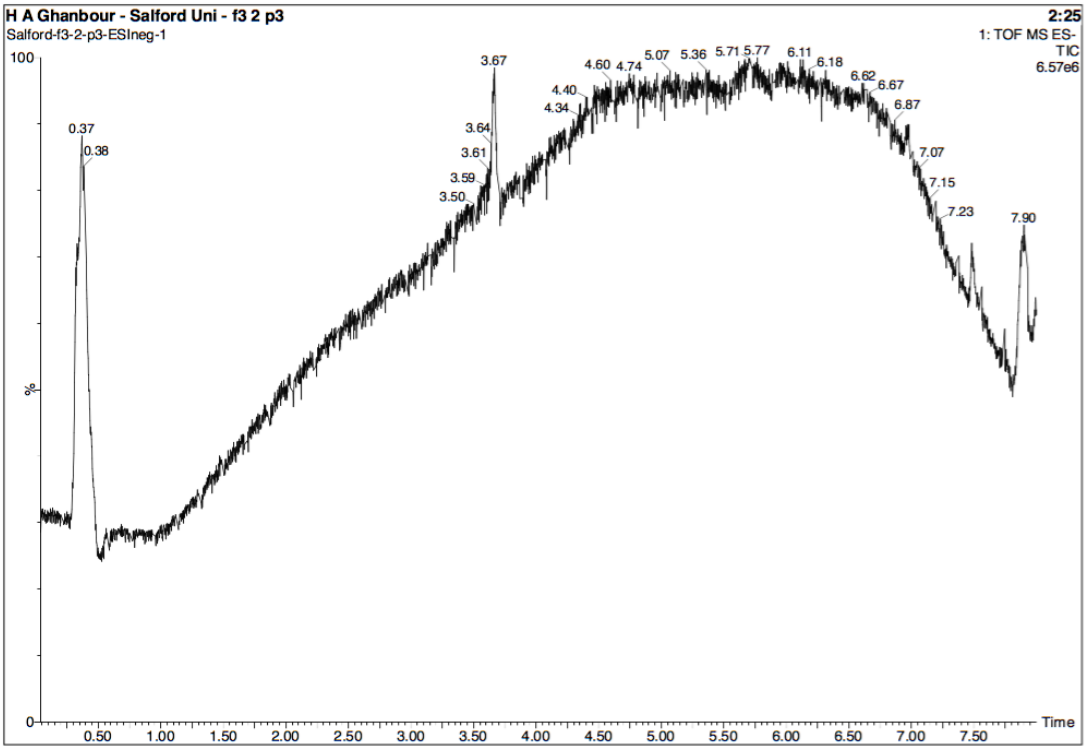
NAME	F3 2 P3
EXPNO	30
PROCNO	1
Date_	20160816
Time	7.59
INSTRUM	spect
PROBHD	5 mm BBO BB-1H
PULPROG	cosygpqf
TD	1440
SOLVENT	MeOD
NS	16
DS	8
SWH	667.735 Hz
FIDRES	0.463705 Hz
AQ	1.0783221 sec
RG	2048
DW	748.800 usec
DE	6.50 usec
TE	296.2 K
D0	0.00000300 sec
D1	0.10000000 sec
D13	0.00000400 sec
D16	0.00010000 sec
IN0	0.00149760 sec
======== CHANNEL f1 ========	
NUC1	1H
P0	11.60 usec
P1	11.60 usec
PL1	2.00 dB
SFO1	400.1330117 MHz
====== GRADIENT CHANNEL =====	
GPNAM1	SINE.100
GP21	10.00 %
P16	1000.00 usec
ND0	1
TD	128
SFO1	400.133 MHz
FIDRES	5.216734 Hz
SW	1.669 ppm
FnMODE	QF
SI	1024
SF	400.1300000 MHz
WDW	SINE
SSB	0
LB	0.00 Hz
GB	0
PC	1.40
SI	1024
MC2	QF
SF	400.1300000 MHz
WDW	SINE
SSB	0
LB	0.00 Hz
GB	0

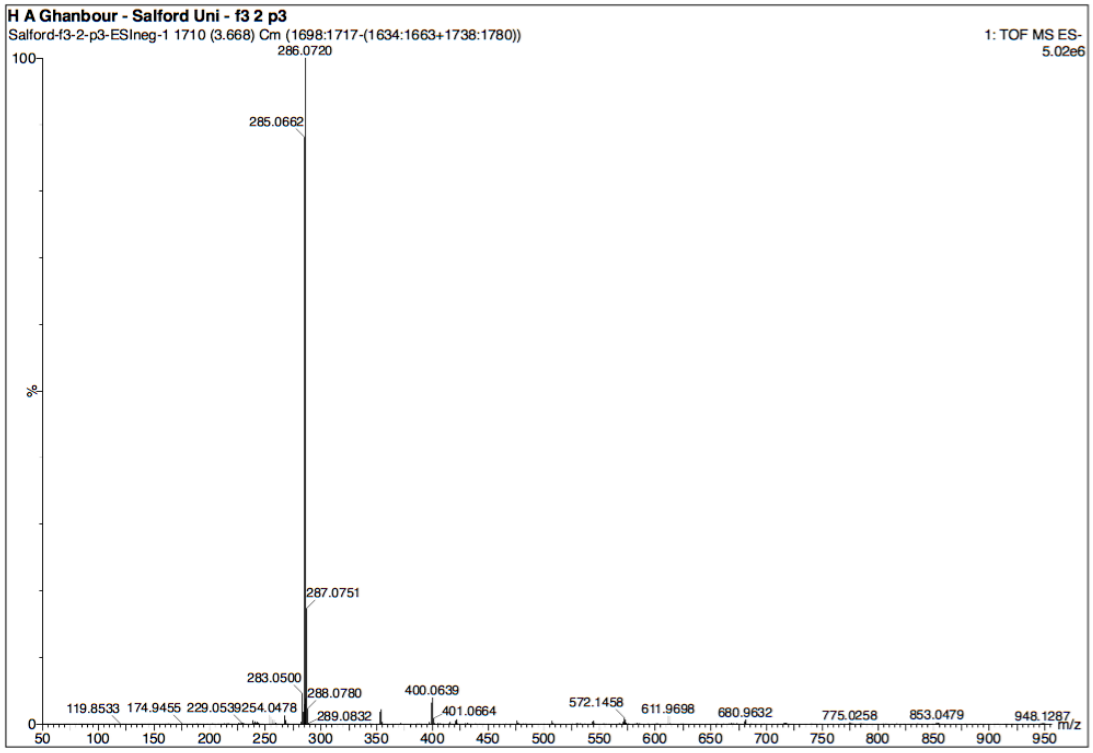
Appendix 3.99: The FTIR spectra (ATR) 4 cm $^{-1}$ (16 Scans) of F3 2 p3



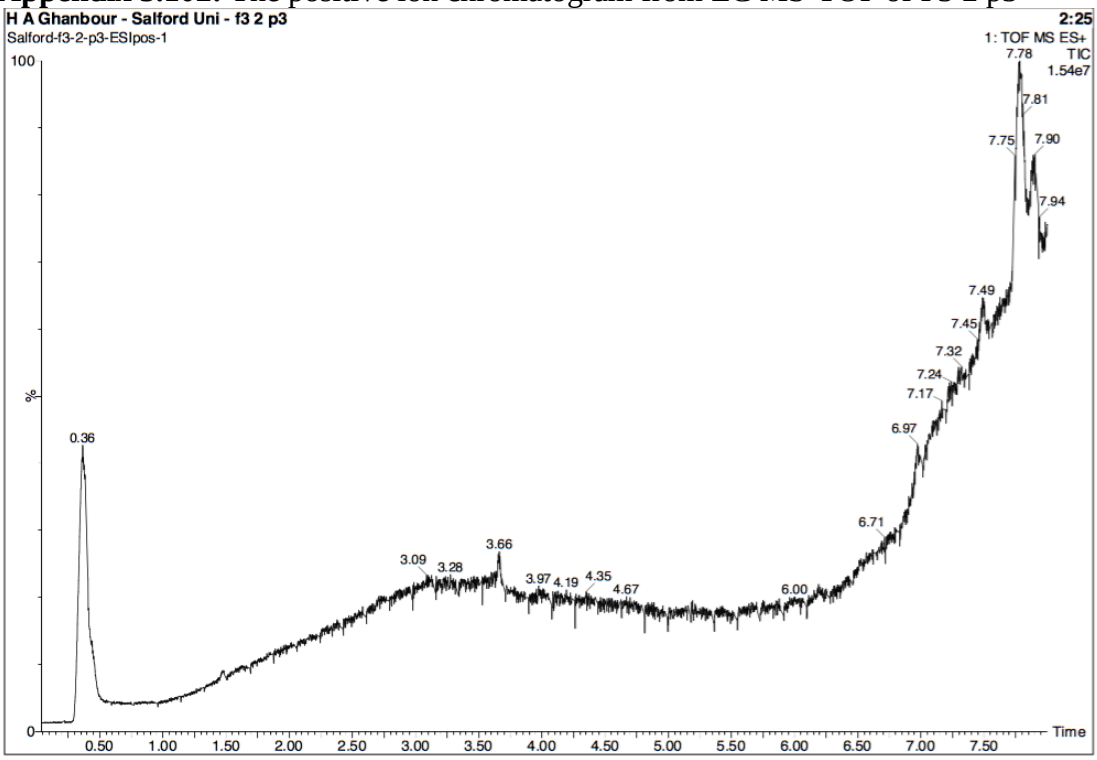
Appendix 3.100: The negative ion chromatogram from LC MS-TOF of F3 2 p3



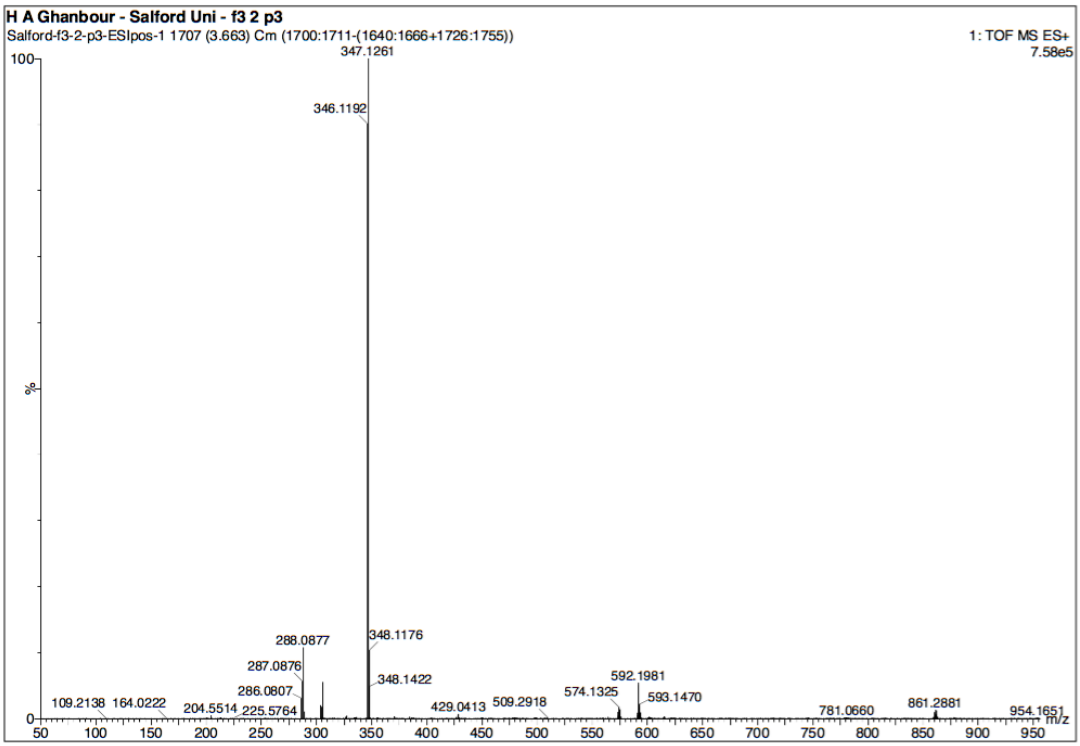
Appendix 3.101: The negative ion spectrum from LC MS-TOF of F3 2 p3



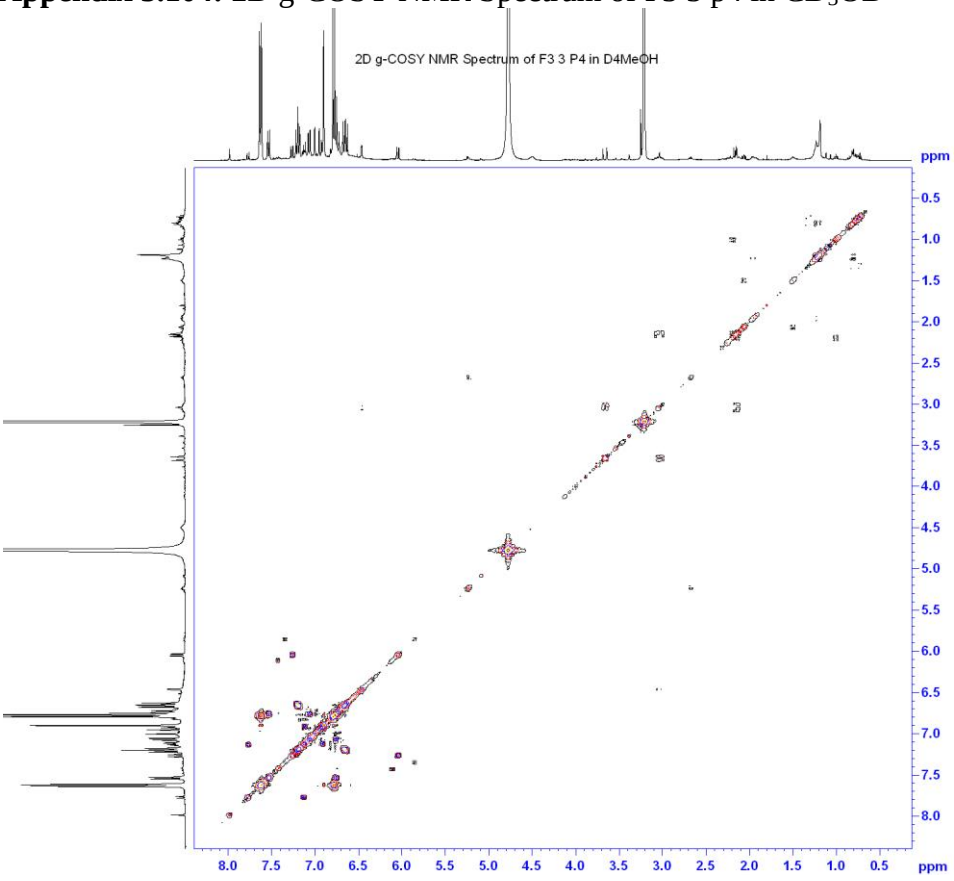
Appendix 3.102: The positive ion chromatogram from LC MS-TOF of F3 2 p3



Appendix 3.103: The positive ion spectrum from LC MS-TOF of F3 2 p3



Appendix 3.104: 2D g-COSY NMR Spectrum of F3 3 p4 in CD_3OD



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```
NAME                 F3 3 P4
EXPNO                   11
PROCNO                   1
Date_             20160720
Time                  8.19
INSTRUM              spect
PROBHD    5 mm BBO BB-1H
PULPROG          cosygpqf
TD                    2048
SOLVENT               MeOD
NS                       8
DS                       8
SWH              3306.878 Hz
FIDRES           1.614687 Hz
AQ               0.3097076 sec
RG                   161.3
DW                151.200 usec
DE                   6.50 usec
TE                  296.2 K
D0               0.00000300 sec
D1               1.36933601 sec
D13              0.00000400 sec
D16              0.00010000 sec
IN0              0.00030240 sec

======== CHANNEL f1 ========
NUC1                    1H
P0                   11.60 usec
P1                   11.60 usec
PL1                   2.00 dB
SFO1          400.1317515 MHz

====== GRADIENT CHANNEL =====
GPNAM1             SINE.100
GPZ1                 10.00 %
D16                1000.00 usec
ND0                      1
TD                     128
SFO1             400.1318 MHz
FIDRES          25.834988 Hz
SW                 8.264 ppm
FnMODE                  QF
SI                    1024
SF            400.1300467 MHz
WDW                  SINE
SSB                     0
LB                   0.00 Hz
GB                      0
PC                   1.40
SI                    1024
MC2                     QF
SF            400.1300467 MHz
WDW                  SINE
SSB                     0
LB                   0.00 Hz
GB                      0
```

Appendix 3.105: BB H1 Decoupled C13 Spectrum of F3 3 p4 in CD_3OD

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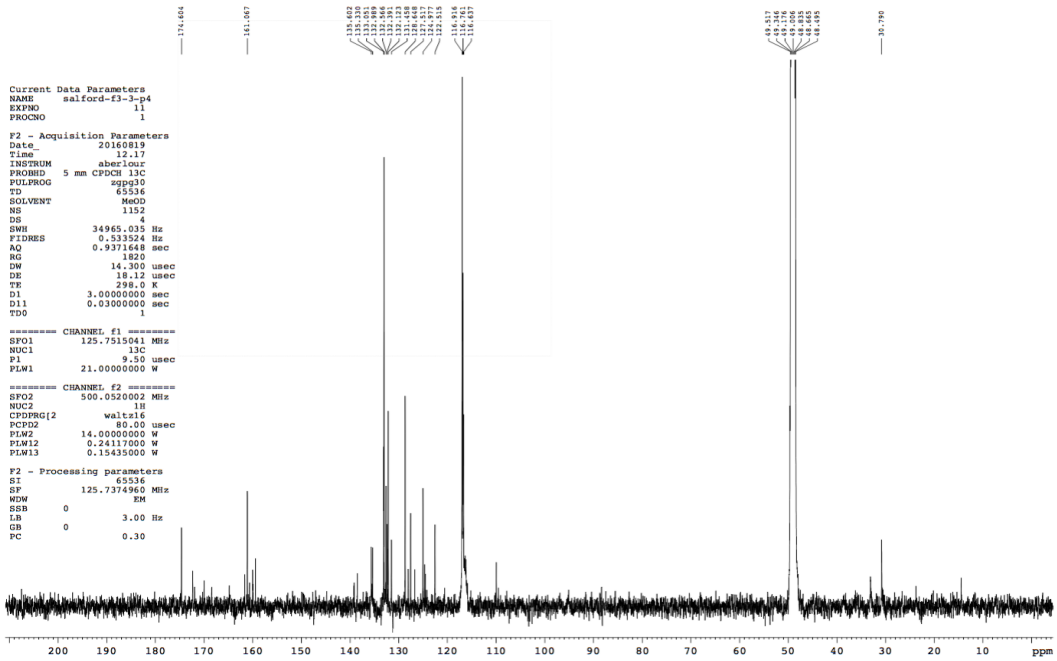
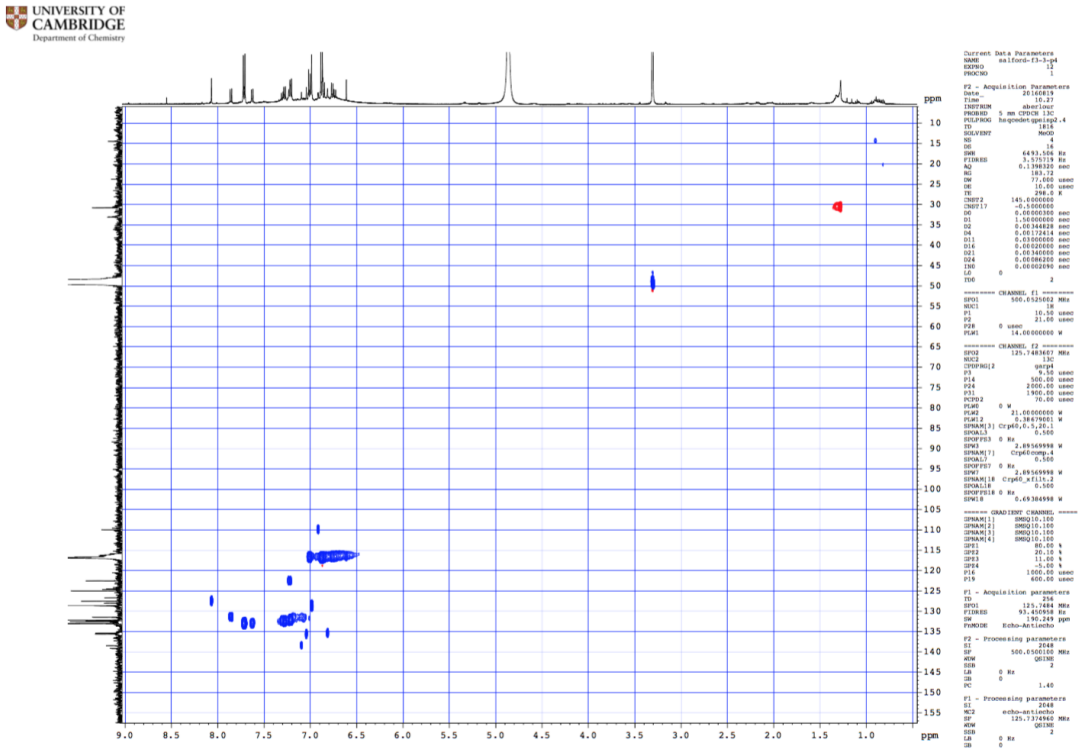
Department of Chemistry

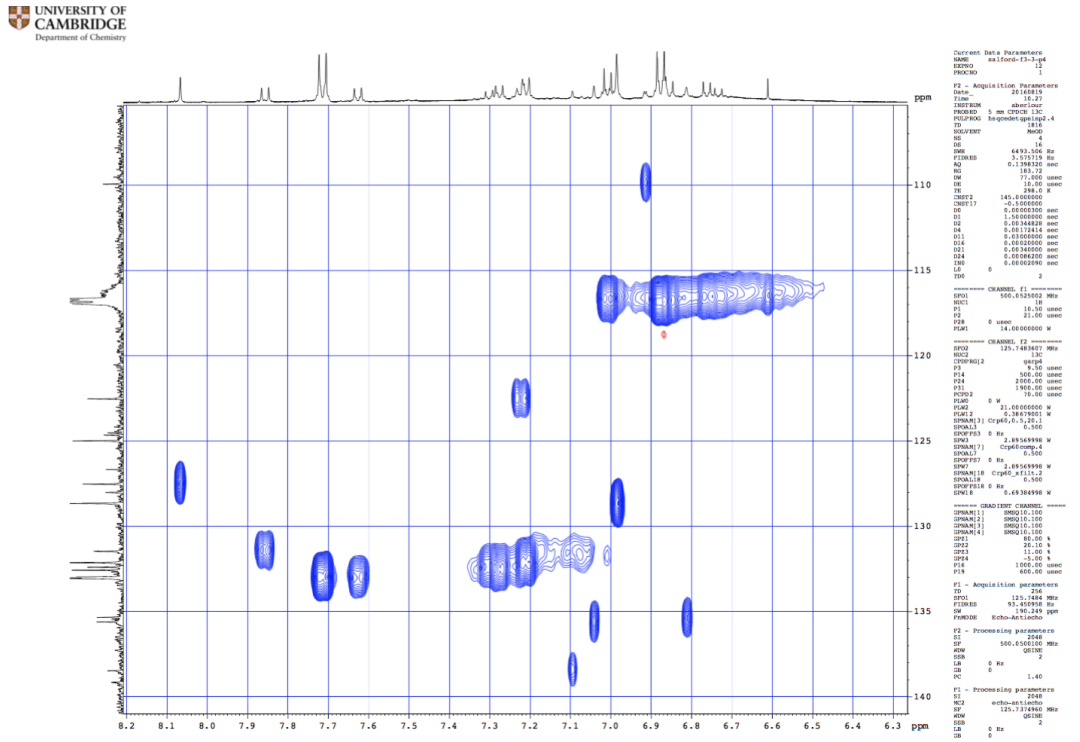



```
Current Data Parameters
NAME      salford-f3-3-p4
EXPNO                  11
PROCNO                  1

F2 - Acquisition Parameters
Date_           20160819
Time               12.17
INSTRUM         aberlour
PROBHD   5 mm CPDCH 13C
PULPROG          zgpg30
TD                65536
SOLVENT            MeOD
NS                 1152
DS                    4
SWH           34965.035 Hz
FIDRES            0.533524 Hz
AQ             0.9371648 sec
RG                 1820
DW                14.300 usec
DE                18.12 usec
TE                298.0 K
D1             3.00000000 sec
D11            0.03000000 sec
TD0                    1

======== CHANNEL f1 ========
SFO1         125.7515041 MHz
NUC1                  13C
P1                   9.50 usec
PLW1        21.00000000 W

======== CHANNEL f2 ========
SFO2         500.0520002 MHz
NUC2                  1H
CPDPRG[2          waltz16
PCPD2               80.00 usec
PLW2        14.00000000 W
PLW12        0.24117000 W
PLW13        0.15435000 W

F2 - Processing parameters
SI                 65536
SF           125.7374960 MHz
WDW                   EM
SSB      0
LB                  3.00 Hz
GB       0
PC                  0.30
```

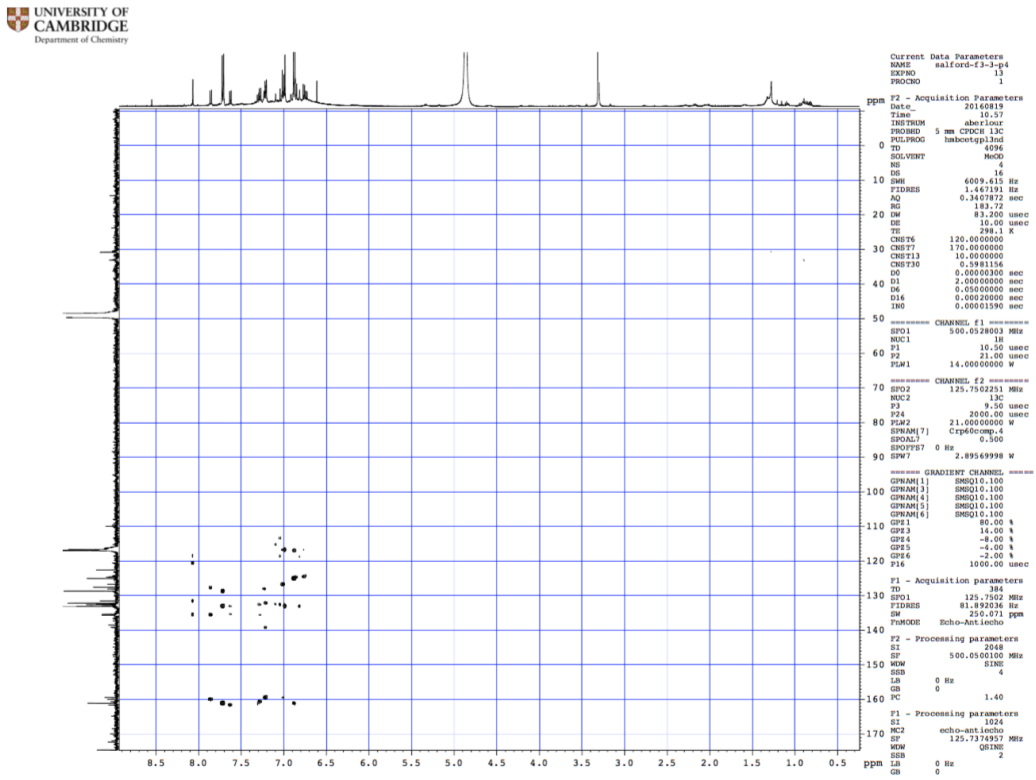
Appendix 3.106: 2 D g-HSQC NMR Spectrum of F3 3 p4 in CD_3OD



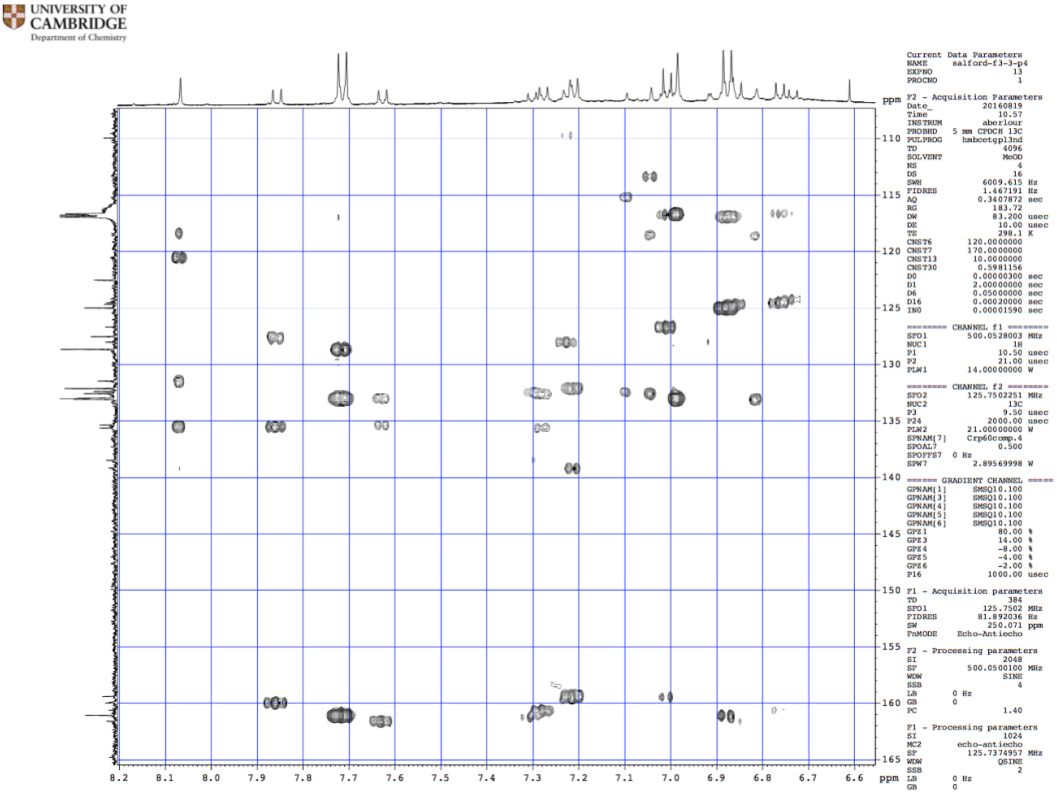
Appendix 3.107: 2 D g-HSQC NMR Spectrum of F3 3 p4 (expansion) in CD_3OD



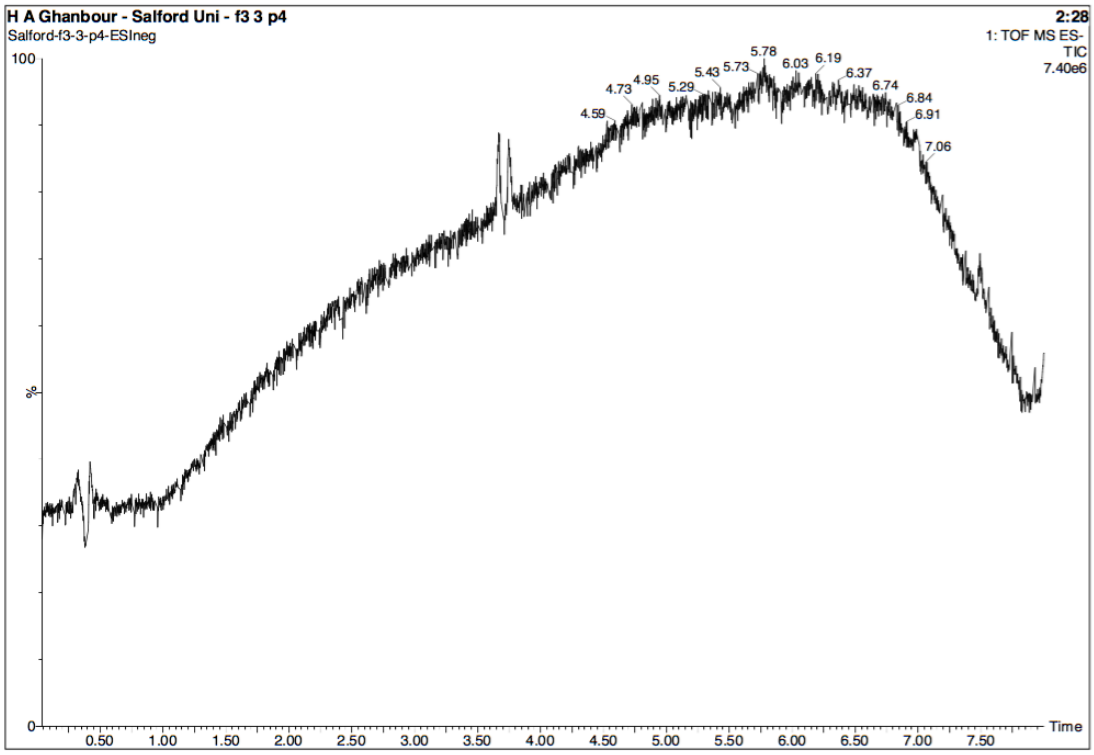
Appendix 3.108: 2 D g-HMBC NMR Spectrum of F3 3 p4 in CD_3OD



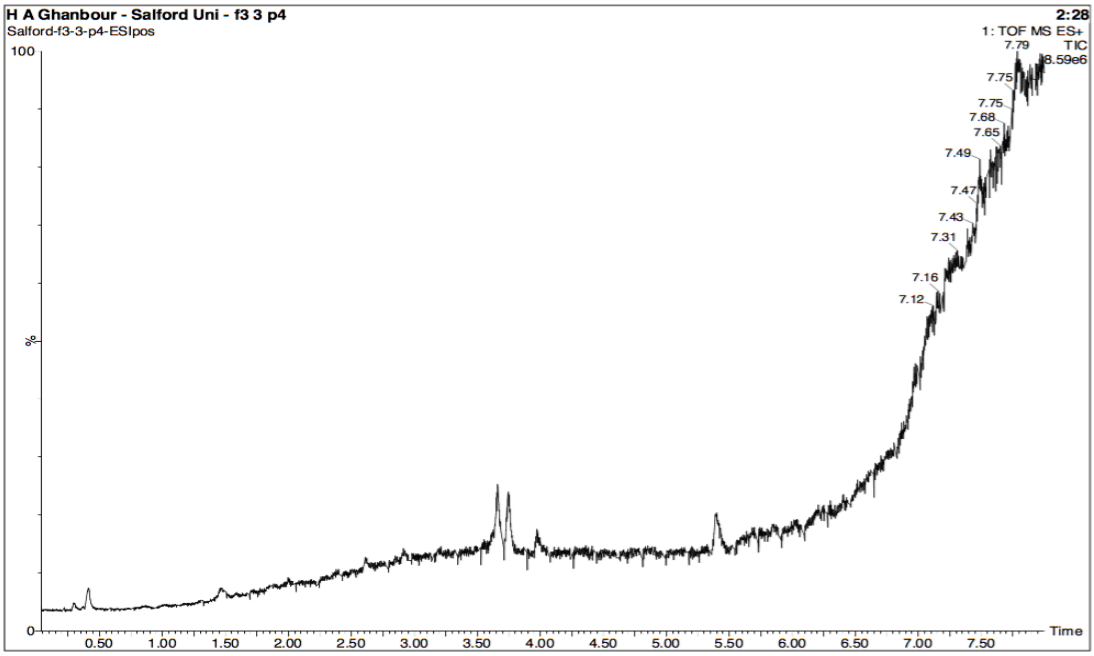
Appendix 3.109: 2 D g-HMBC NMR Spectrum of F3 3 p4 (expansion) in CD_3OD



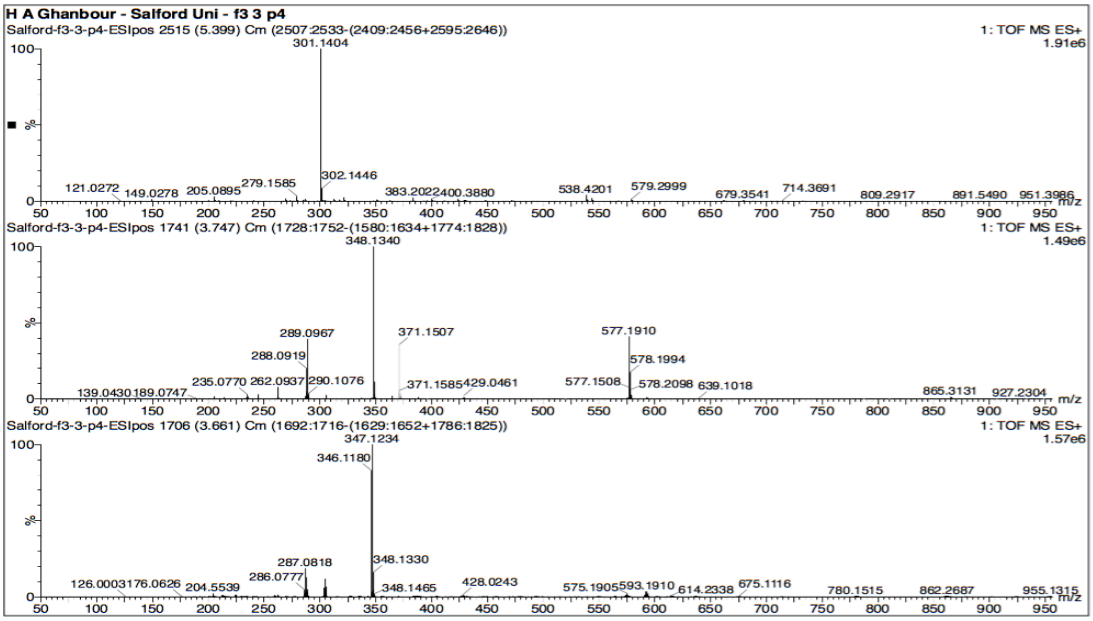
Appendix 3.110: The negative ion chromatogram from LC MS-TOF of F3 3 p4



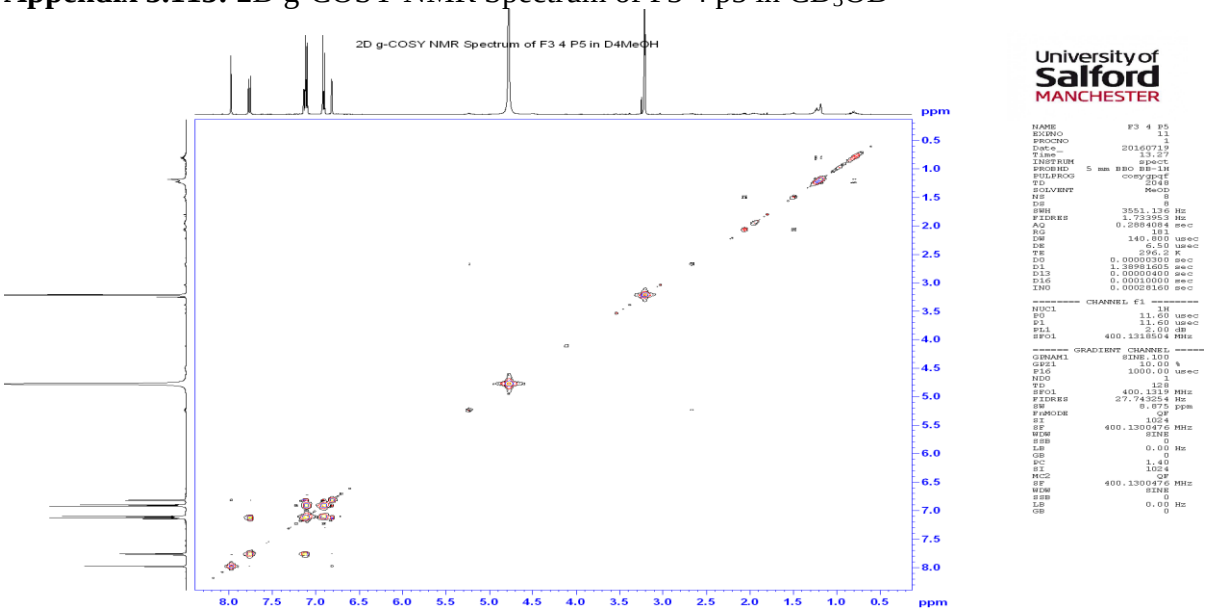
Appendix 3.111: The positive ion chromatogram from LC MS-TOF of F3 3 p4



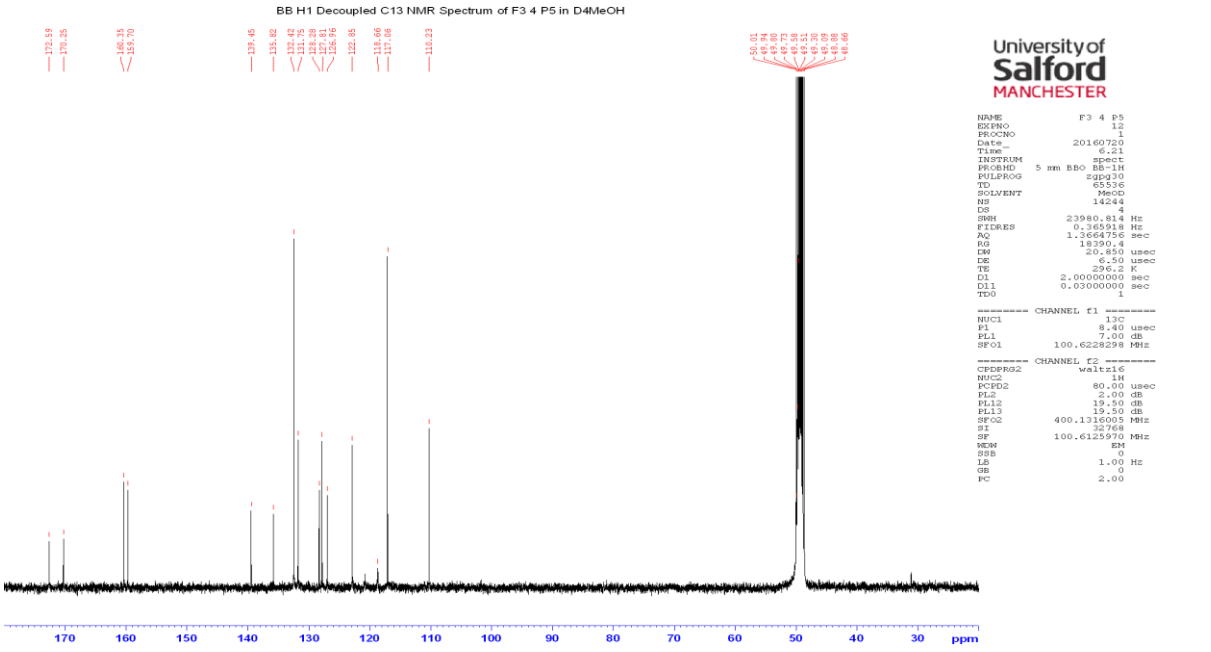
Appendix 3.112: The positive ion spectrum from LC MS-TOF of F3 3 p4



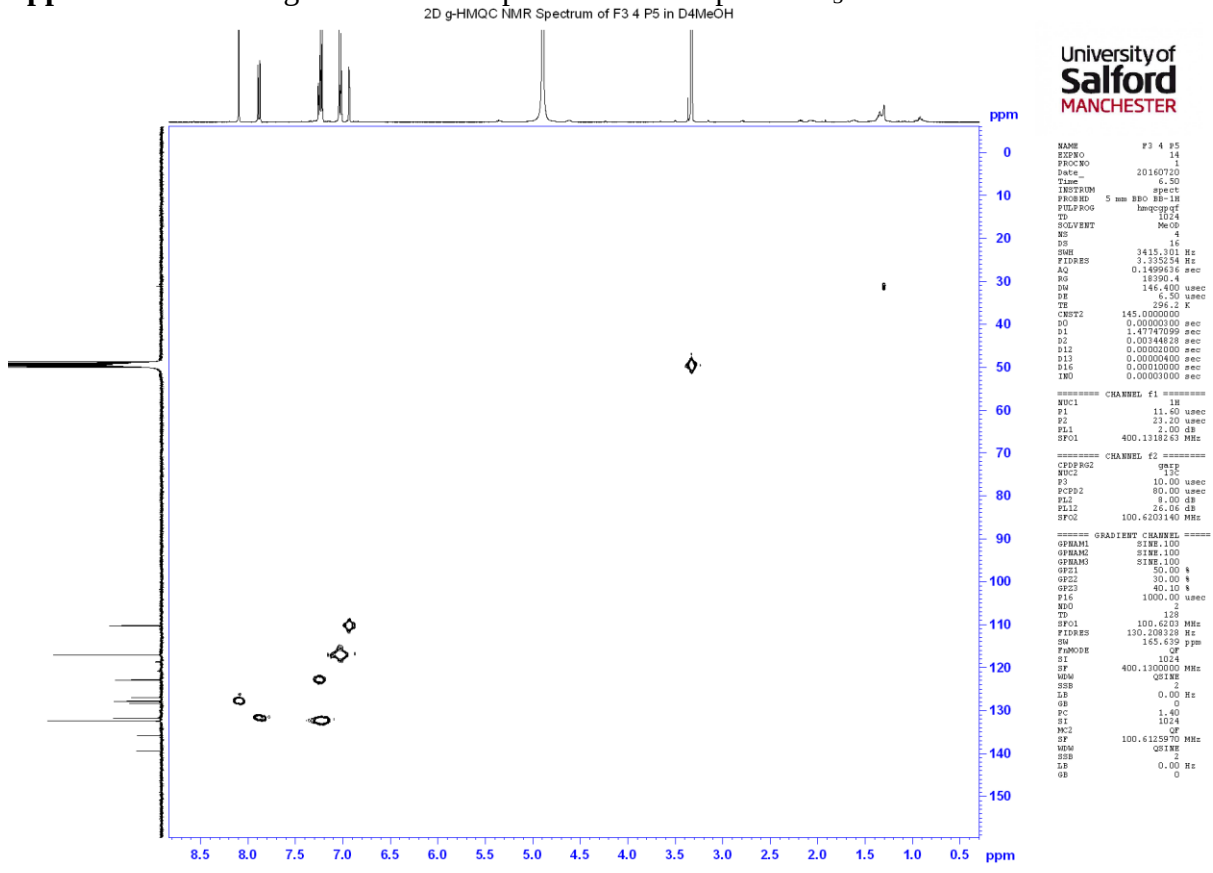
Appendix 3.113: 2D g-COSY NMR Spectrum of F3 4 p5 in CD_3OD



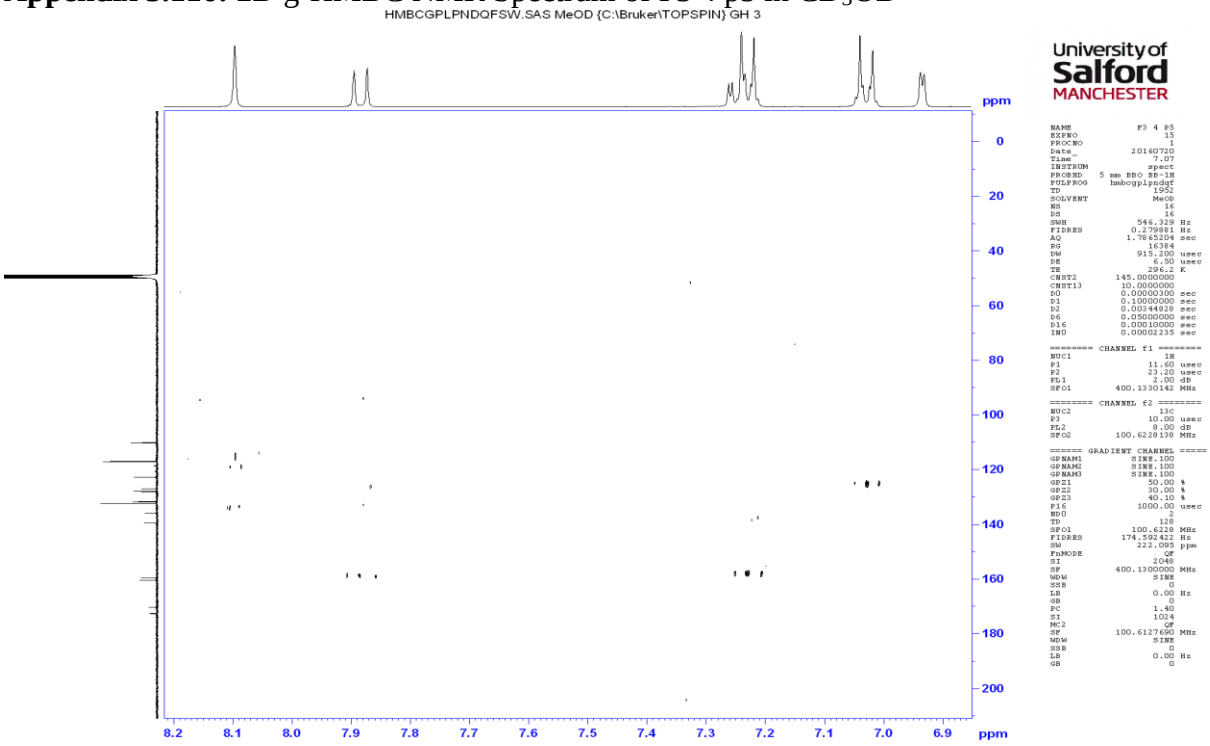
Appendix 3.114: BB H1 Decoupled C13 Spectrum of F3 4 p5 in CD_3OD



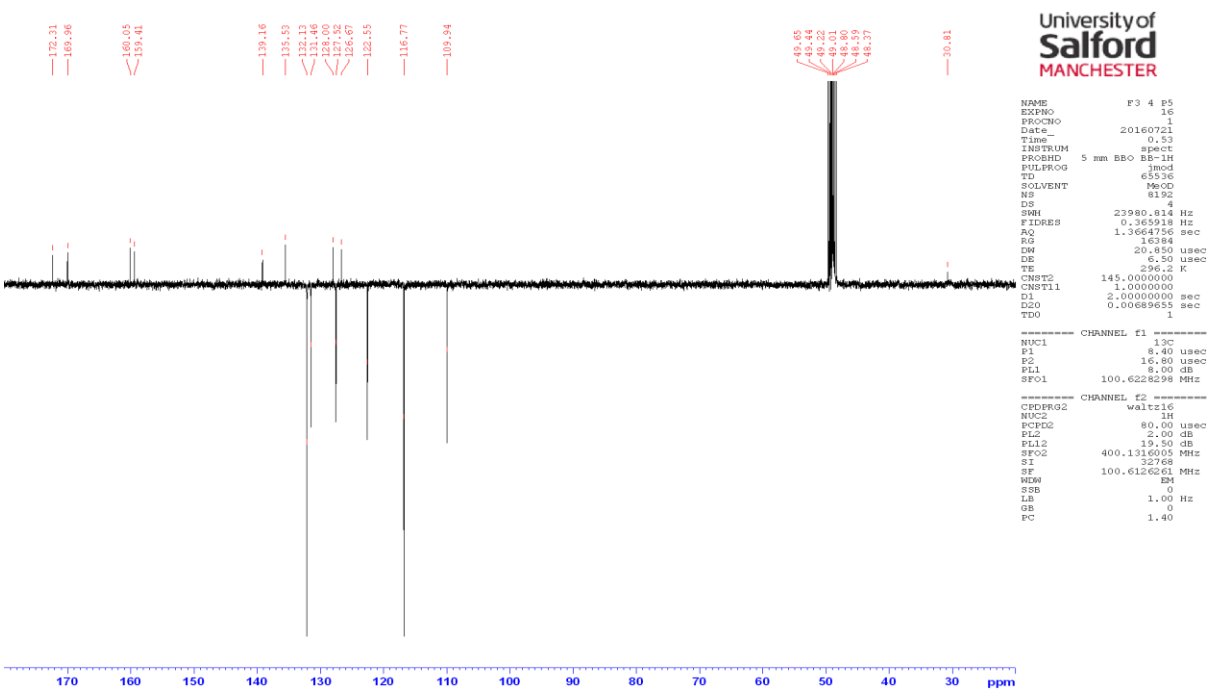
Appendix 3.115: 2D g-COSY NMR Spectrum of F3 4 p5 in CD_3OD



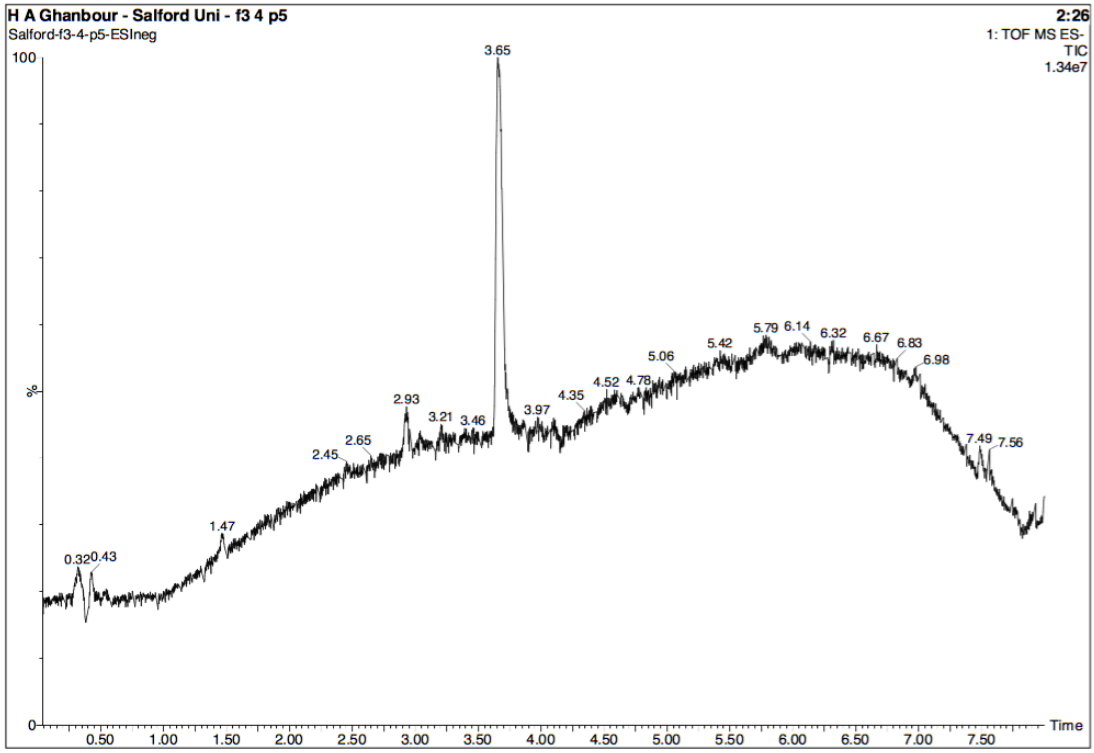
Appendix 3.116: 2D g-HMBC NMR Spectrum of F3 4 p5 in CD_3OD



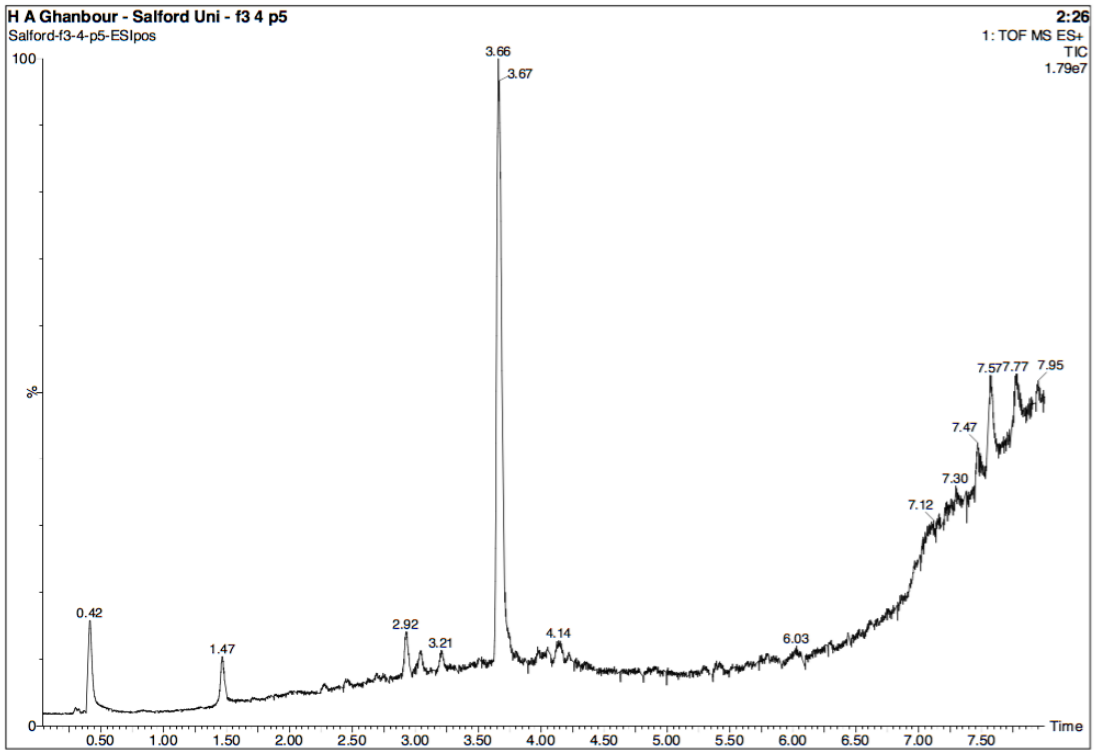
Appendix 3.117: JMOD C13 Spectrum of F3 4 p5 in CD_3OD



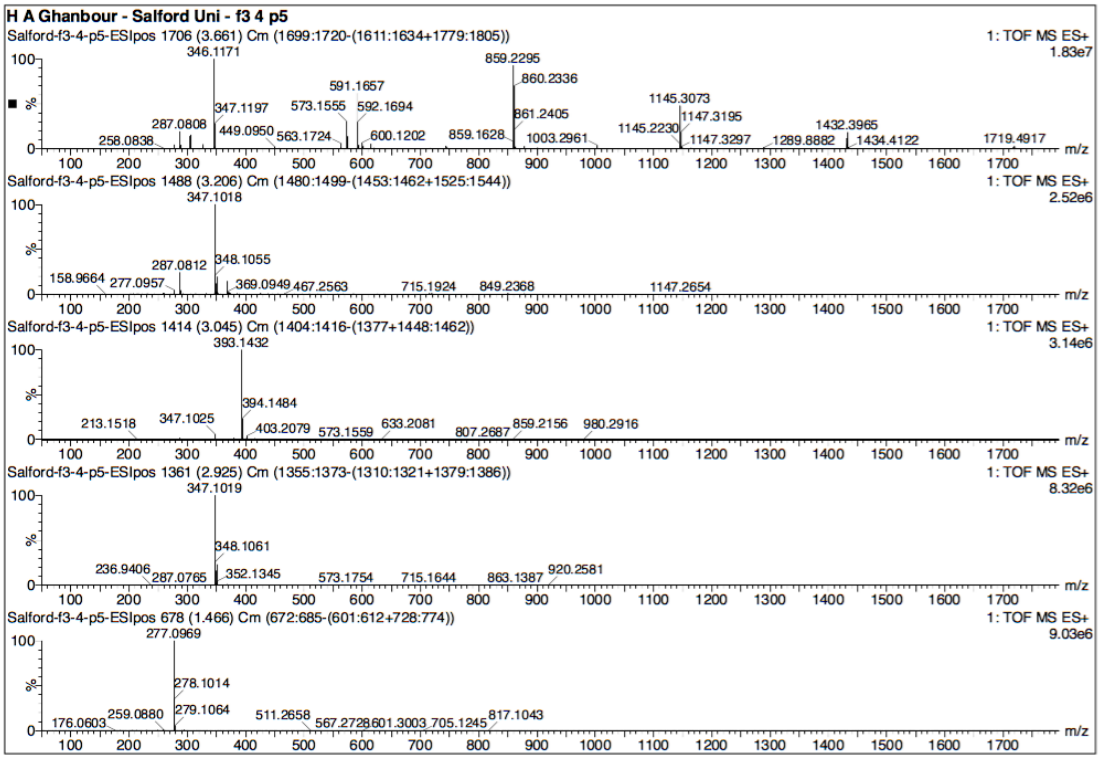
Appendix 3.118: The negative ion chromatogram from LC MS-TOF of F3 4 p5



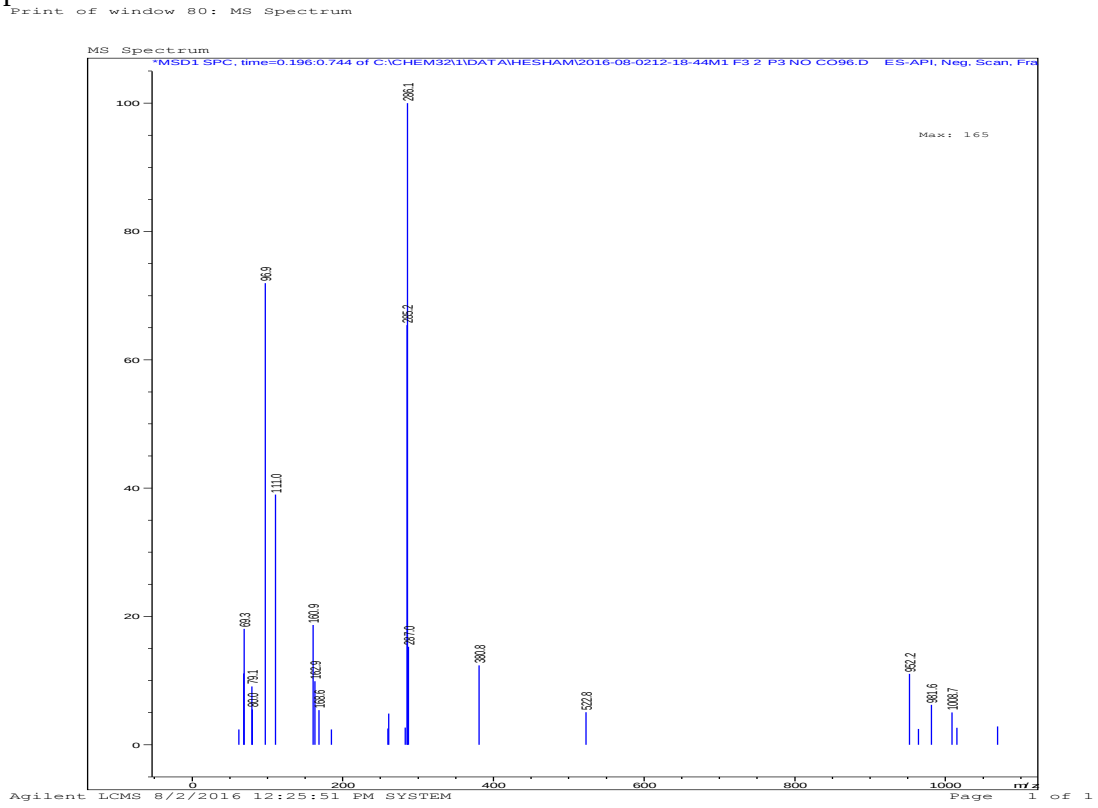
Appendix 3.119: The positive ion chromatogram from LC MS-TOF of F3 4 p5



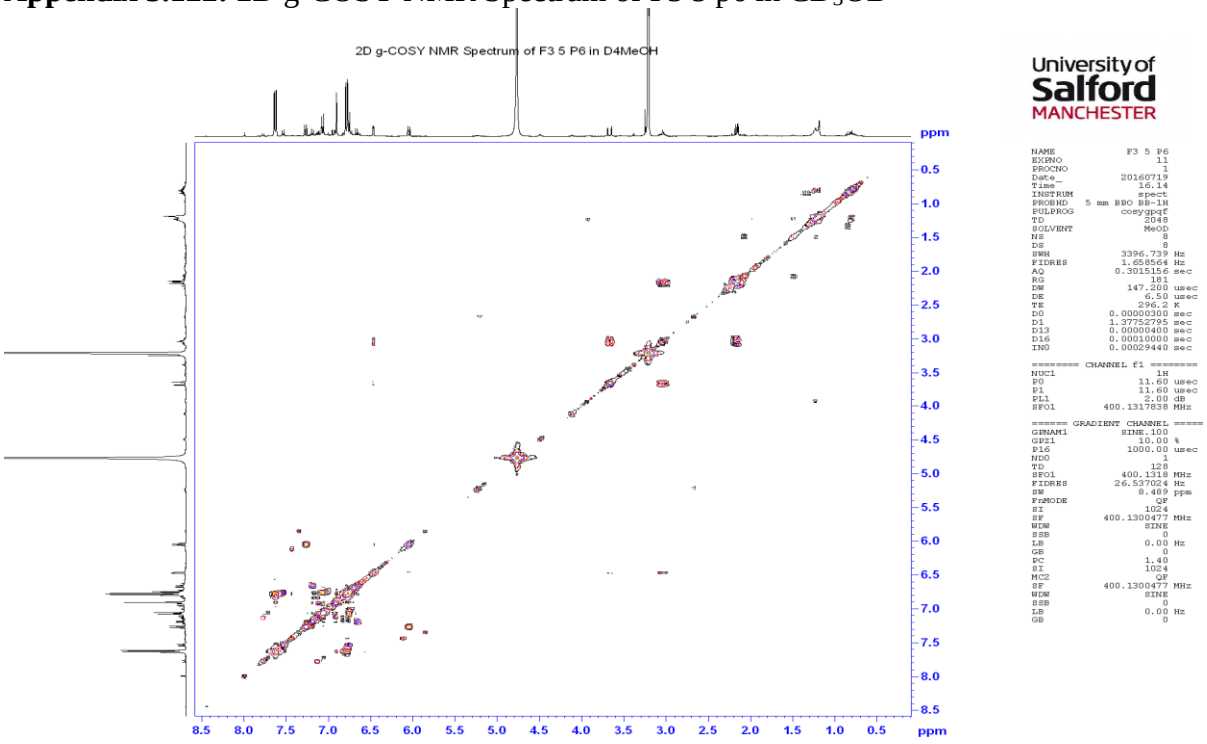
Appendix 3.120: The positive ion spectrum from LC MS-TOF of F3 4 p5



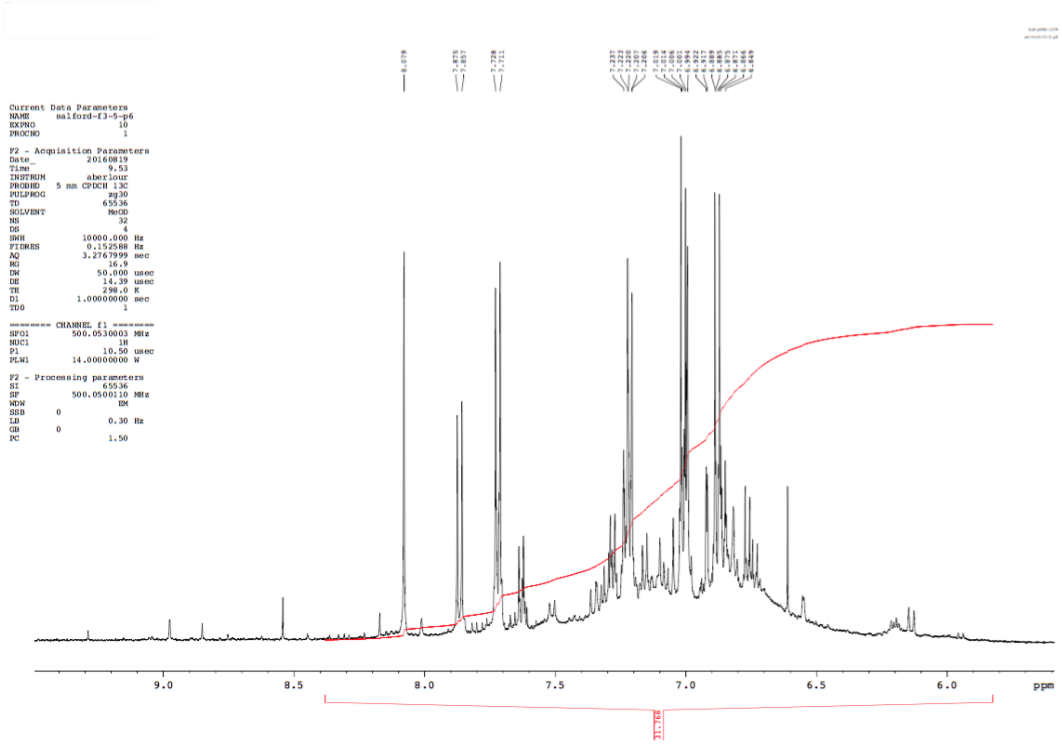
Appendix 3.121: The negative ion spectrum from LC MS (Salford University) of F3 4 p5



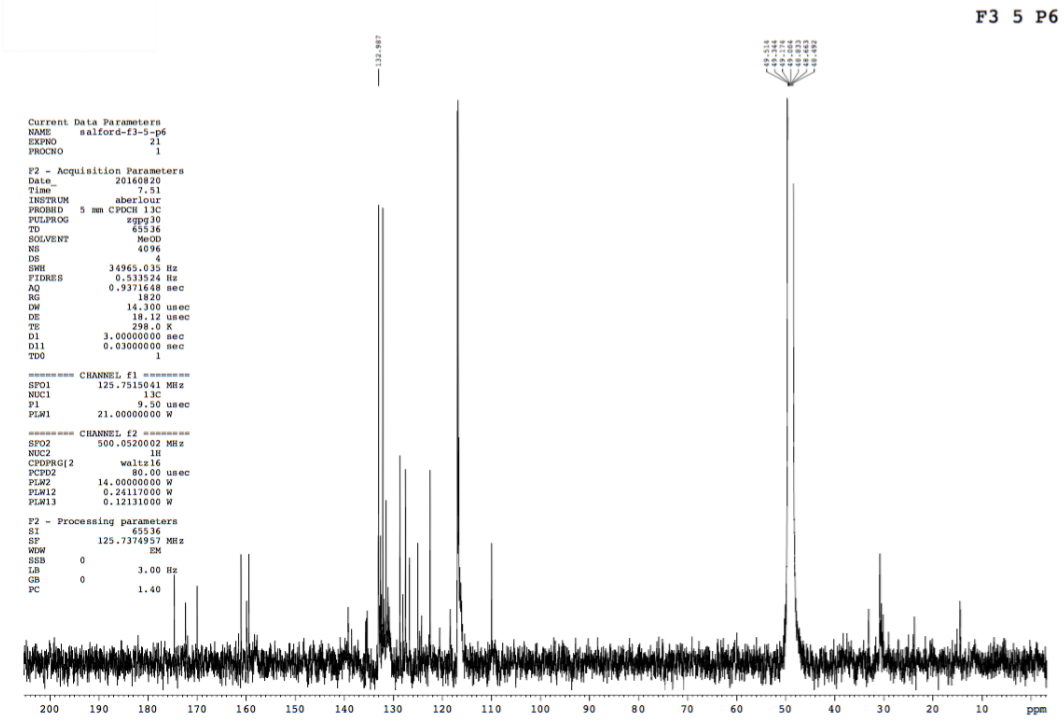
Appendix 3.122: 2D g-COSY NMR Spectrum of F3 5 p6 in CD_3OD



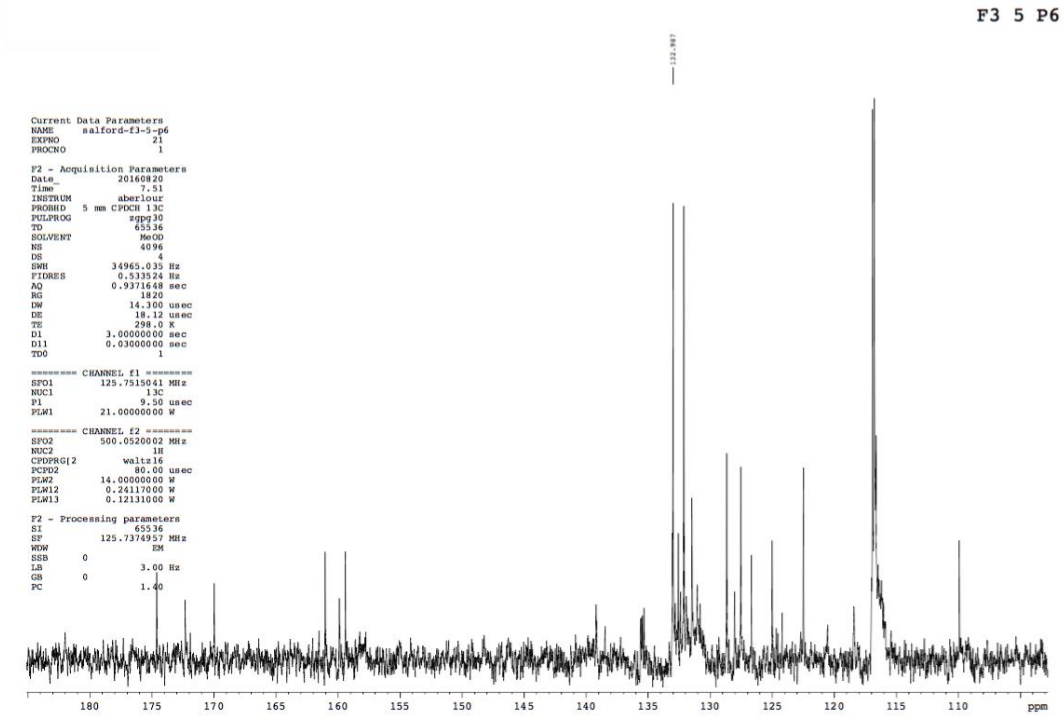
Appendix 3.123: ^{1}H NMR Spectrum of F3 5 p6 (expansion) in CD_3OD+TMS



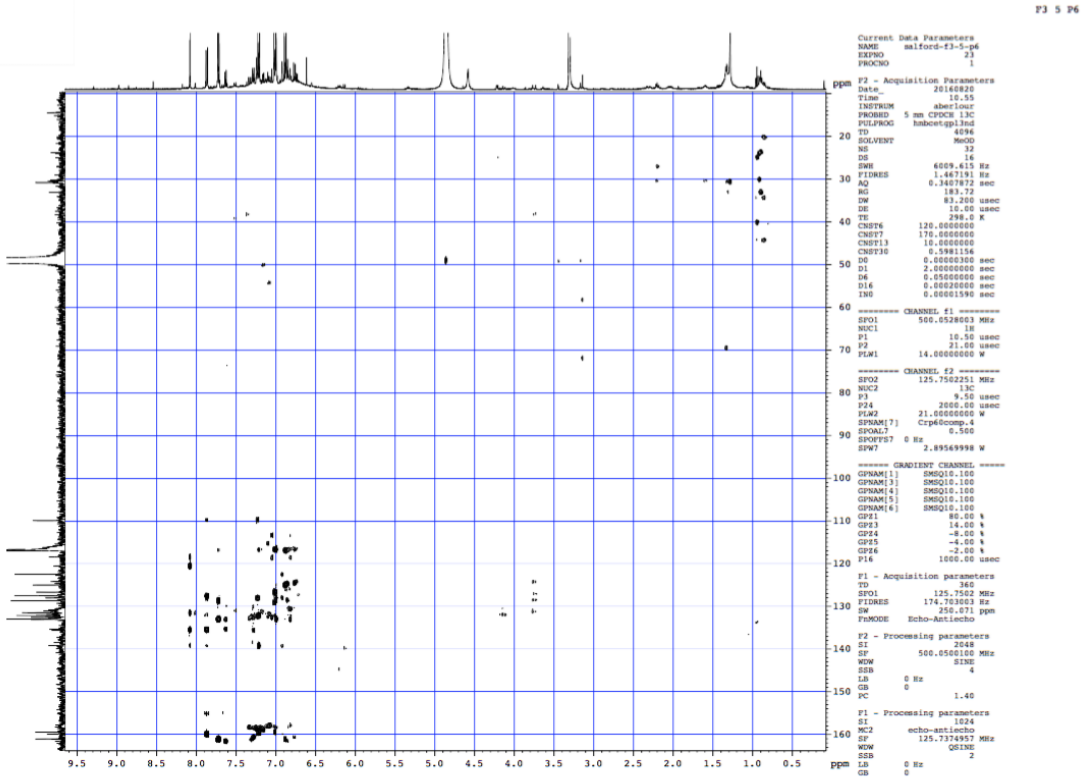
Appendix 3.124: BB H1 Decoupled C13 Spectrum of F3 5 p6 in CD_3OD



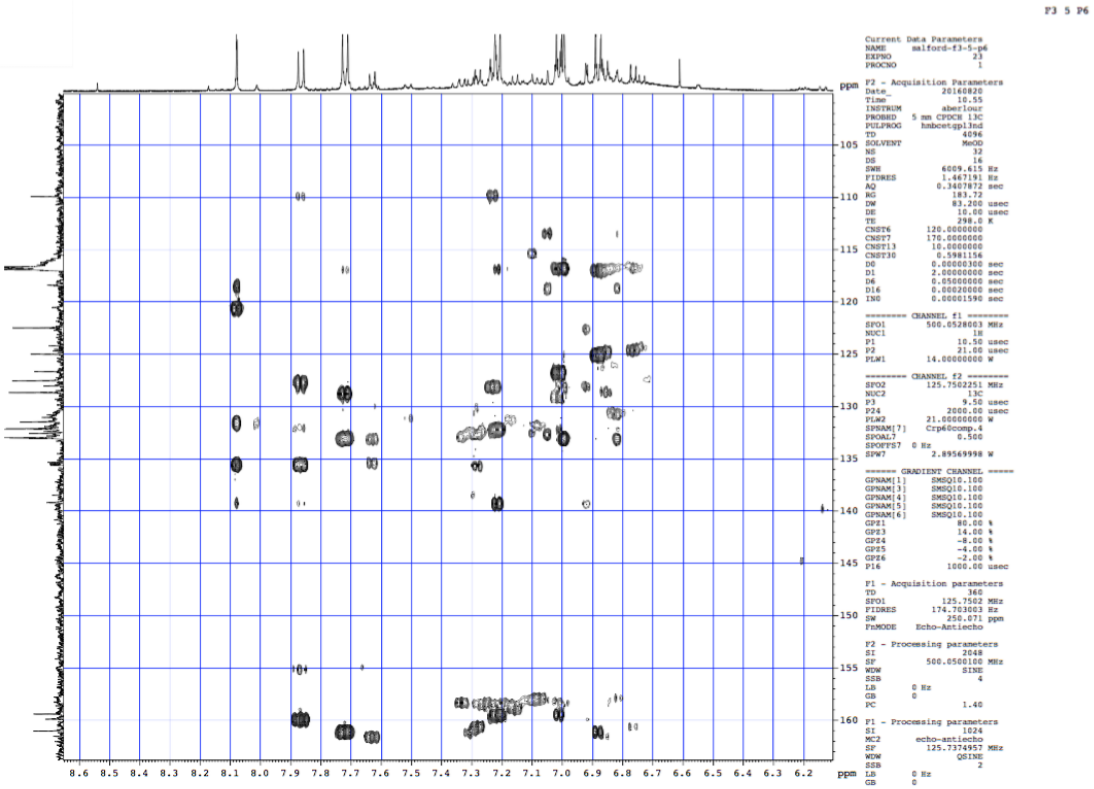
Appendix 3.125: BB H1 Decoupled C13 Spectrum of F3 5 p6 (expansion) in CD_3OD



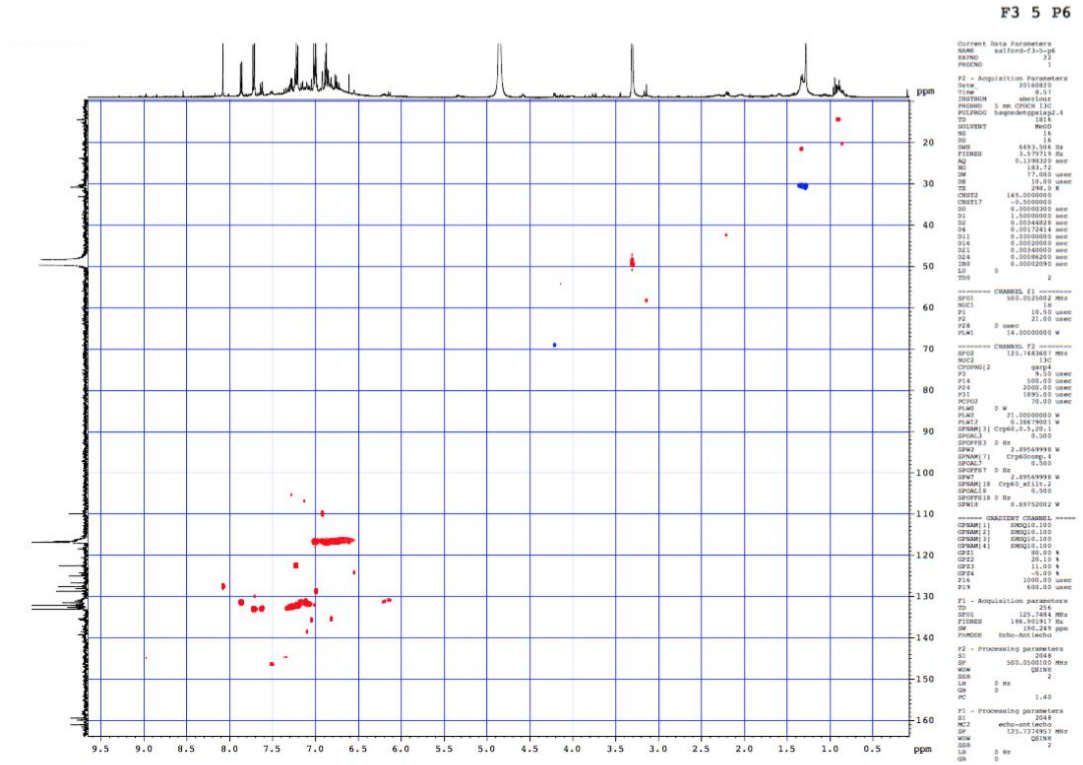
Appendix 3.126: 2 D g-HMBC NMR Spectrum of F3 5 p6 in CD_3OD



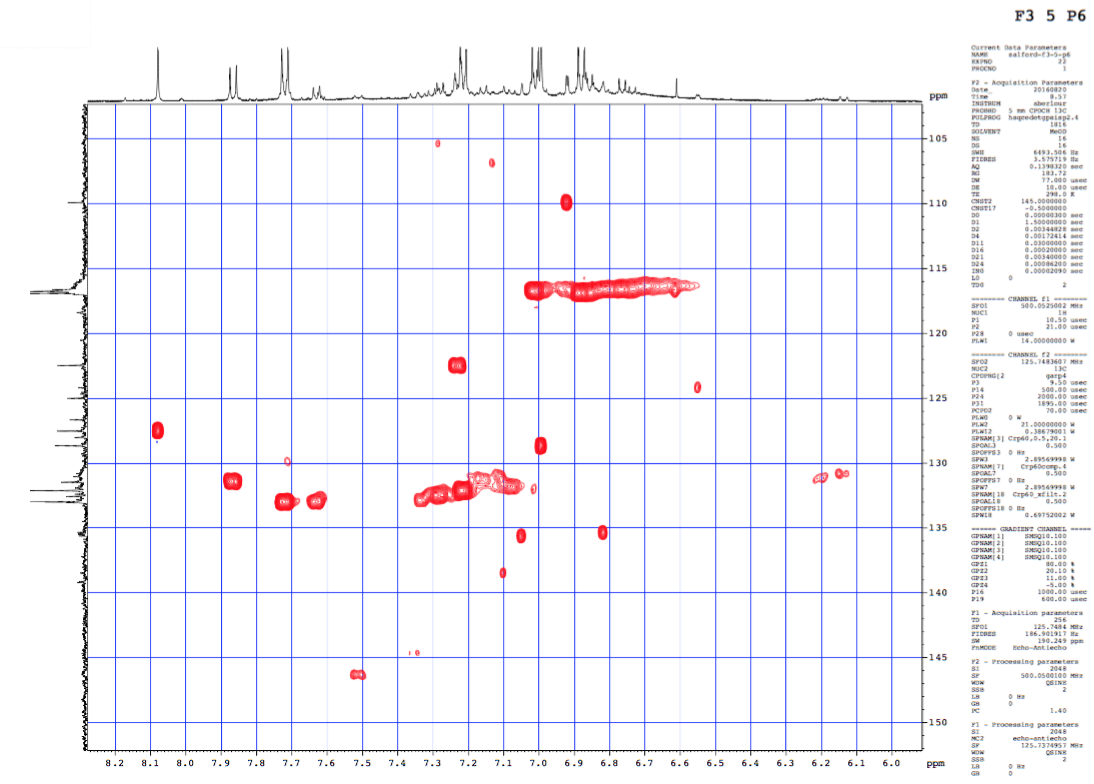
Appendix 3.127: 2 D g-HMBC NMR Spectrum of F3 5 p6 (expansion) in CD_3OD



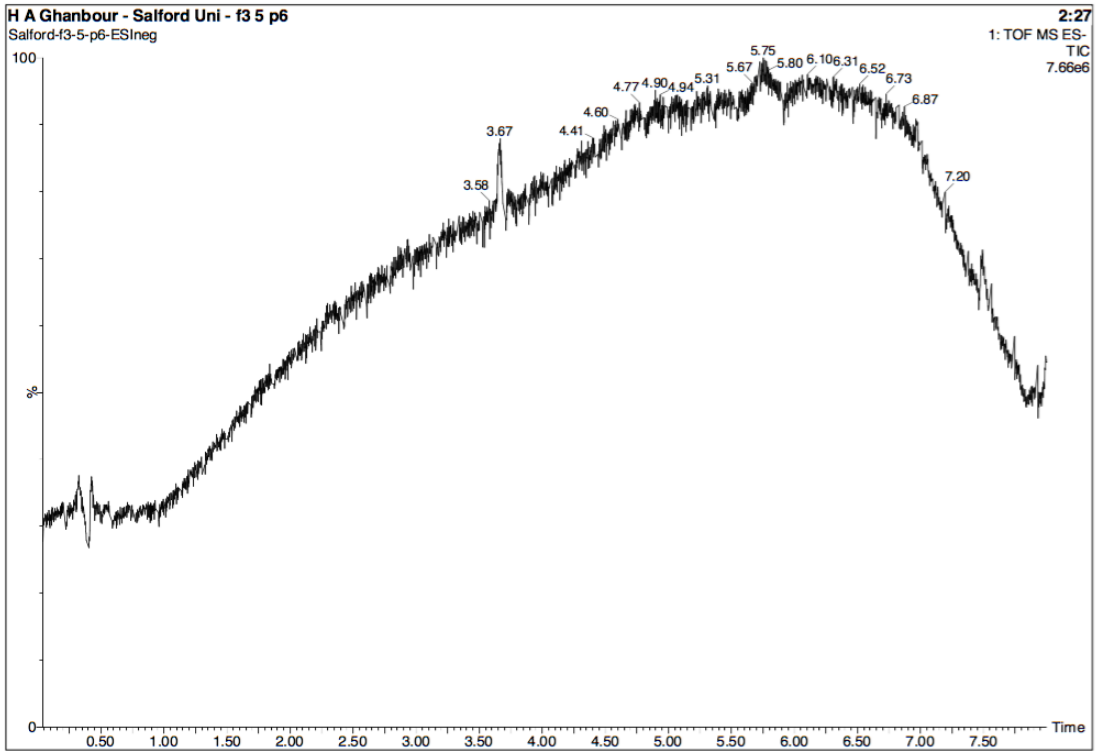
Appendix 3.128: 2 D g-HSQC NMR Spectrum of F3 5 p6 in CD_3OD



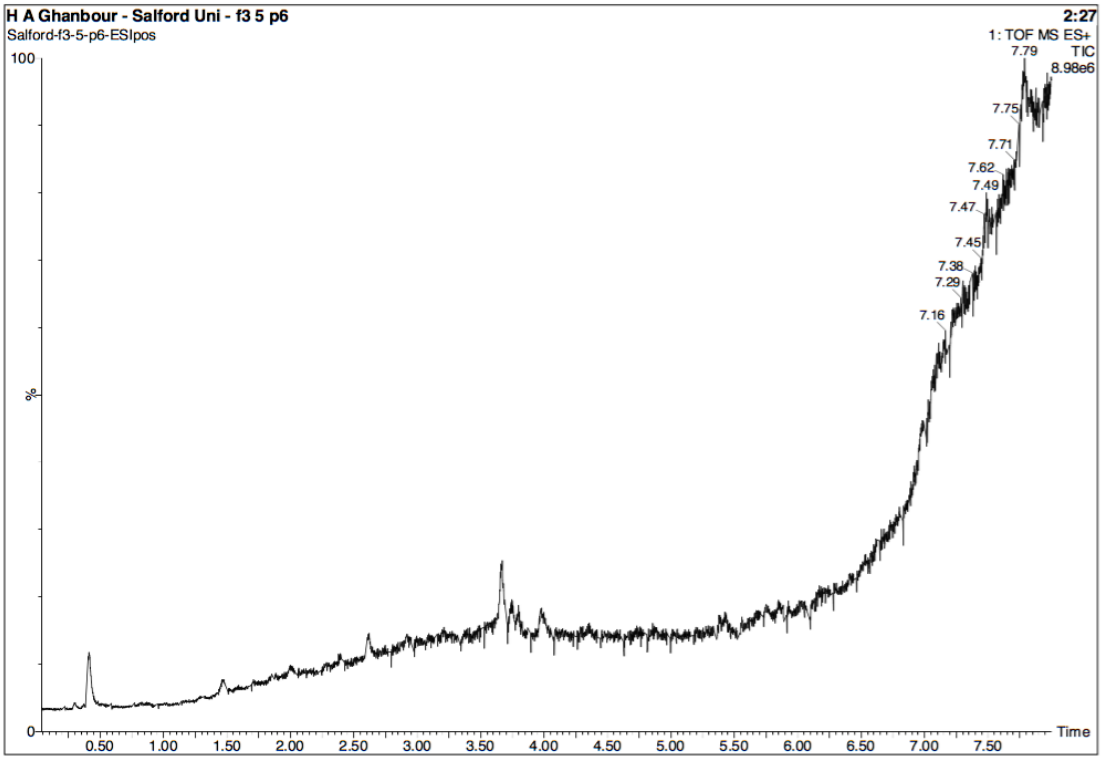
Appendix 3.129: 2 D g-HSQC NMR Spectrum of F3 5 p6 (expansion) in CD_3OD



Appendix 3.130: The negative ion chromatogram from LC MS-TOF of F3 5 p6



Appendix 3.131: The positive ion chromatogram from LC MS-TOF of F3 5 p6



Appendix 3.132: The positive ion spectrum from LC MS-TOF of F3 5 p6